

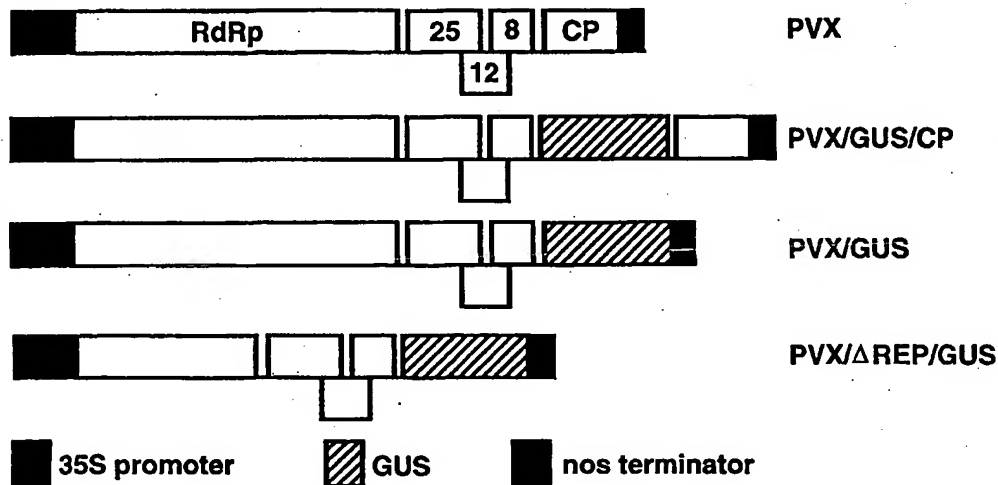
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(54) Title: METHODS AND MEANS FOR GENE SILENCING IN TRANSGENIC PLANTS



(57) Abstract

Disclosed are DNA constructs ("amplicons" TM) comprising a promoter operably linked to DNA which can be transcribed in a plant cell to an RNA transcript, which RNA transcript includes plant virus sequence (e.g. derived from potato virus X) which confers on the RNA transcript the ability to replicate in the cytoplasm of the plant cell. The constructs may lack portions of the viral genome not required for such replication (e.g. coat proteins) but preferably include targeting sequence which is capable of down-regulating (for instance gene silencing) expression of one or more target genes, which may be endogenous plant genes, transgenes, or pathogen genes. Also disclosed are corresponding vectors, host cells, plants (particularly having modified and/or silenced genes or being pathogen resistant) and plant products, plus also methods of producing and using these materials.

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METHODS AND MEANS FOR GENE SILENCING
IN TRANSGENIC PLANTS

The present invention relates to "gene silencing" ("gs") in transgenic plants. It employs "amplicon constructs", providing in various aspects nucleic acid molecules and vectors, cells and plants containing these, and methods and uses.

"Gene silencing" is a term generally used to refer to suppression of expression of a gene. The degree of reduction may be so as to totally abolish production of the encoded gene product, but more usually the abolition of expression is partial, with some degree of expression remaining. The term should not therefore be taken to require complete "silencing" of expression. It is used herein where convenient because those skilled in the art well understand this.

Transgenes may be used to suppress endogenous plant genes. This was discovered originally when chalcone synthase transgenes in petunia caused suppression of the endogenous chalcone synthase genes (29,35). Subsequently it has been described how many, if not all plant genes can be "silenced" by transgenes (11,12,16,17,19,23).

Gene silencing requires sequence similarity between the transgene and the gene that becomes silenced (Matzke, M. A. and Matzke, A. J. M. (1995), *Trends in Genetics*, 11: 1-3). This sequence homology may involve promoter regions or coding regions of the silenced gene (Matzke, M. A. and Matzke, A. J. M. (1993) *Annu. Rev. Plant*

Physiol. Plant Mol. Biol., 44: 53-76, Vaucheret, H.

(1993) *C. R. Acad. Sci. Paris*, 316: 1471-1483, Vaucheret,

H. (1994), *C. R. Acad. Sci. Paris*, 317: 310-323,

Baulcombe, D. C. and English, J. J. (1996), *Current*

5 *Opinion In Biotechnology*, 7: 173-180, Park, Y-D., et al

(1996); *Plant J.*, 9: 183-194). When coding regions are

involved the transgene able to cause gene silencing may

have been constructed with a promoter that would

transcribe either the sense or the antisense orientation

10 of the coding sequence RNA. In at least one example the

coding sequence transgene was constructed without a

promoter (Van Blokland, R., et al (1994), *Plant J.*, 6:

861-877). It is likely that the various examples of gene

silencing involve different mechanisms that are not well

15 understood. In different examples there may be

transcriptional or post transcriptional gs (3,4,10,24).

It has also become clear that gene silencing can
account for some characteristics of transgenic plants

that are not easily reconciled with conventional

20 understanding of genetics. For example the wide

variation in transgene expression between sibling lines

with a transgene construct is due in part to gene

silencing: low expressers are those with a high level of

gene silencing whereas high expressers are those in which

25 gene silencing is absent or induced late in plant

development (10,13,14). Similarly gene silencing can

often explain virus resistance in transgenic lines in

which a viral cDNA transgene is expressed at a low level:

the gene silencing mechanism acting on RNA inhibits accumulation of both the transgene RNA and the viral RNA (5,10,22).

In principle there is an enormous practical potential of gs for crop improvement. It is possible to silence genes conferring unwanted traits in the plant by transformation with transgene constructs containing elements of these genes. Examples of this type of application include gs of ripening specific genes in tomato to improve processing and handling characteristics of the harvested fruit; gs of genes involved in pollen formation so that breeders can reproducibly generate male sterile plants for the production of F1 hybrids; gs of genes involved in lignin biosynthesis to facilitate paper making from vegetative tissue of the plant; gs of genes involved in flower pigment production to produce novel flower colours; gs of genes involved in regulatory pathways controlling development or environmental responses to produce plants with novel growth habit or (for example) disease resistance; elimination of toxic secondary metabolites by gs of genes required for toxin production. In addition, gs is can be useful as a means of developing virus resistant plants when the transgene is similar to a viral genome.

A major complication in the practical exploitation of this phenomenon to date is the unpredictable and low occurrence of gs. Typically there will be strong gs in as few as 5-20% of lines generated with any one construct

(for examples see (28,34)). Therefore, it has not been realistic to attempt gs in plants that are difficult to transform and for which it is difficult to produce many transformants. Similarly, it would be difficult to
5 activate gs against several different traits or against several viruses in the same plant. Even with plants that are easy to transform the need to generate multiple lines limits the ease of exploitation of gs.

The first indication that an inoculated virus could
10 elicit gene silencing was with transgenic plants in which the transgene included cDNA of tobacco etch potyvirus (22). The lines that exhibited this virus-induced gene silencing were initially high level expressers of the transgene. After inoculation with a strain of tobacco
15 etch potyvirus that was identical or highly similar to the transgene there was a reduction in the amount of the transgene RNA and suppression of the originally inoculated virus in the upper leaves of the plant. These upper leaves were described as having recovered because
20 they were virus and symptom free. They were also resistant against a secondary challenge inoculation with virus that was highly similar to the transgene at the nucleotide level. All of these effects are probably due to gs at the post transcriptional level.

25 However, there is nothing in this report to indicate that virus-induced gene silencing is intrinsically more reproducible than any other type of gene silencing. In fact there were some lines that

displayed the virus-induced gene silencing and other lines that did not (22). Furthermore there are many reports that refer to virus inoculation of transgenic plants carrying transgenes that are similar or identical to the inoculated virus. Of these reports only a minority describe virus-induced gene silencing. Thus with viral transgenes the virus induced gene silencing is the exception rather than the rule. That is, as indicated above, there was no indication from this work that gene silencing by inoculated viruses is intrinsically reproducible.

Biosource Technologies, Inc. (20,21) have suggested the use of genetic constructions based on RNA viruses which replicate in the cytoplasm of cells to provide inhibitory RNA, either anti-sense or co-suppressor RNA. Cells are transfected with the cytoplasmically-replicating genetic constructions in which the RNA encoding region is specific for the gene of interest. Experimental evidence illustrating the drawbacks and limitations of the Biosource approach is included below.

The present invention aims to overcome one or more of the many problems in the art. For instance, experimental evidence included below demonstrates with embodiments of the present invention gs is achievable more reproducibly than with conventional technology.

Briefly, the present invention in various aspects makes use of an amplicon construct which will exhibit gs targeted against sequence with homology to a sequence

within the amplicon. An amplicon is a transgene DNA construct including a promoter and cDNA of at least part of a viral genome, and optionally a transcriptional terminator. Preferably, the construct includes a
5 "targeting sequence", which may be a sequence foreign to the virus, for specifically targeting down-regulation of a gene of interest ("target gene"). Further details are discussed below.

Incorporation of a construct in the genome of
10 transgenic plants in accordance with the present invention may be used to ensure the viral cDNA is transcribed from the promoter in many or most cells of the plant, though use of a tissue- or developmentally-regulated and/or inducible promoter is possible. If the
15 viral cis-acting elements and trans-acting factors necessary for replication are intact there will be replication of the viral RNA in the transgenic plant. As
a consequence, either direct or indirect, there will be activation of gs targeted against sequences with
20 sufficient homology to a sequence included with the replicating viral RNA, the "targeting sequence".

An amplicon construct may have an unmodified viral cDNA. In such a case the plant may be resistant against the virus as a result of gs. Other amplicon constructs
25 may have a foreign targeting sequence inserted into the viral cDNA and the gs will be targeted against the corresponding sequence as well as against the virus. The targeting sequence may be part of a different viral

genome, in which case the plant may also be resistant against this second virus. The targeting sequence may be derived from a nuclear gene or transgene, or a gene on an extrachromosomal element such as a plasmid, and the gs
5 targeted against that gene and homologues.

The present invention will now be discussed in more detail.

According to one aspect of the present invention there is provided, preferably within a vector suitable
10 for stable transformation of a plant cell, a DNA construct in which a promoter is operably linked to DNA for transcription in a plant cell of an RNA molecule which includes plant virus sequences which confer on the RNA molecule the ability to replicate in the cytoplasm of
15 a plant cell following transcription. The RNA transcribed from the DNA which is under the transcriptional control of the promoter is capable of replication in the cytoplasm of a plant cell by virtue of including appropriate plant virus sequences. The
20 transcripts, possibly including a sequence foreign to the virus, replicate as if they are viral RNAs, activating gs.

The transcribed RNA generally includes a sequence ("targeting sequence") which is complementary to a
25 sequence in a target gene, either in the sense or anti-sense orientation, or a sequence which has sufficient homology to a target sequence for down-regulation of expression of the target gene to occur. Whilst not to be

bound by any theory, it is believed that sense and anti-sense regulation involve hybridisation between sequences which are sufficiently complementary to hybridise under conditions within a cell.

5 The targeting sequence within the construct may be foreign to the plant virus, i.e. of or derived from a gene or sequence which the virus lacks. Those skilled in the art will understand that terms such as "exogenous" or "heterologous" may equally be used in this context.

10 A vector which contains the construct may be used in transformation of one or more plant cells to introduce the construct stably into the genome, so that it is stably inherited from one generation to the next. This is preferably followed by regeneration of a plant from
15 such cells to produce a transgenic plant.

 Thus, in further aspects, the present invention also provides the use of the construct or vector in production of a transgenic plant, methods of transformation of cells and plants, plant and microbial
20 (particularly *Agrobacterium*) cells, and various plant products.

 The function of the promoter in the amplicon construct is to ensure that the DNA is transcribed into RNA containing the viral sequences. By "promoter" is
25 meant a sequence of nucleotides from which transcription may be initiated of DNA operably linked downstream (i.e. in the 3' direction on the sense strand of double-stranded DNA). A promoter "drives" transcription of an

operably linked sequence.

"Operably linked" means joined as part of the same nucleic acid molecule, suitably positioned and oriented for transcription to be initiated from the promoter. DNA
5 operably linked to a promoter is "under transcriptional initiation regulation" of the promoter, or "in functional combination" therewith.

Preferred promoters may include the 35S promoter of cauliflower mosaic virus or the nopaline synthase
10 promoter of *Agrobacterium tumefaciens* (Sanders, P. R., et al (1987), *Nucleic Acids Res.*, 15: 1543-1558). These promoters are expressed in many, if not all, cell types of many plants. Other constitutively expressed promoters may be used effectively as components of amplicon
15 construct producing gs. Depending on the target gene of amplicon gs, other promoters including those that are developmentally regulated or inducible may be used. For example, if it is necessary to silence the target gene specifically in a particular cell type the amplicon
20 construct may be assembled with a promoter that drives transcription only in that cell type. Similarly, if the target gene is to be silenced following a defined external stimulus the amplicon construct may incorporate a promoter that is be activated specifically by that
25 stimulus. Promoters that are both tissue specific and inducible by specific stimuli may be used.

Inducible promoters may be advantageous in certain circumstances because they place the timing of reduction

in expression of the target gene of interest under the control of the user.

The term "inducible" as applied to a promoter is well understood by those skilled in the art. In essence, expression under the control of an inducible promoter is "switched on" or increased in response to an applied stimulus. The nature of the stimulus varies between promoters. Some inducible promoters cause little or undetectable levels of expression (or no expression) in the absence of the appropriate stimulus. Other inducible promoters cause detectable constitutive expression in the absence of the stimulus. Whatever the level of expression is in the absence of the stimulus, expression from any inducible promoter is increased in the presence of the correct stimulus. The preferable situation is where the level of expression increases upon application of the relevant stimulus by an amount effective to alter a phenotypic characteristic. Thus an inducible (or "switchable") promoter may be used which causes a basic level of expression in the absence of the stimulus which level is too low to bring about a desired phenotype (and may in fact be zero). Upon application of the stimulus, expression is increased (or switched on) to a level which brings about the desired phenotype.

Suitable promoters include the Cauliflower Mosaic Virus 35S (CaMV 35S) gene promoter that is expressed at a high level in virtually all plant tissues (Benfey et al, 1990a and 1990b) and the maize glutathione-S-transferase

isoform II (GST-II-27) gene promoter which is activated in response to application of exogenous safener (WO93/01294, ICI Ltd). The GST-II-27 gene promoter has been shown to be induced by certain chemical compounds which can be applied to growing plants. The promoter is functional in both monocotyledons and dicotyledons. It can therefore be used to control gene expression in a variety of genetically modified plants, including field crops such as canola, sunflower, tobacco, sugarbeet, cotton; cereals such as wheat, barley, rice, maize, sorghum; fruit such as tomatoes, mangoes, peaches, apples, pears, strawberries, bananas, and melons; and vegetables such as carrot, lettuce, cabbage and onion. The GST-II-27 promoter is also suitable for use in a variety of tissues, including roots, leaves, stems and reproductive tissues.

A construct for use in accordance with the present invention includes sequences which are DNA copies of cis-acting elements in the viral genome and of open reading frames in the viral genome that are required for replication of the viral RNA. They should be arranged in the amplicon construct so that the transcripts of the amplicon construct will replicate in the plant cells independently of other transgenes or of viruses inoculated to the plants. The cDNA copies of any other parts of the viral genome need not be included in the amplicon construct provided that their absence does not interfere with replication of the amplicon transcripts.

An optional but important additional feature of some amplicon constructs in accordance with the present invention is the insertion of foreign sequences into the viral cDNA. The site of insertion of these foreign sequences is so that the foreign sequence is replicated, as RNA, as part of the viral RNA produced by transcription of the amplicon in a plant cell. The target of gene silencing is determined by the viral cDNA and any foreign targeting sequence in the amplicon construct.

The amplicon constructs used in the experimental exemplification described in this application for the purpose of illustration of aspects of the present invention without limitation are based on cDNA of the potato virus X (PVX) genome (Kavanagh, T. A., et al (1992), *Virology*, 189: 609-617). Many, if not all, other RNA or DNA viruses of plants may be used in generation of amplicon constructs in a manner that is similar to that described here for PVX. Particularly suitable alternatives to PVX are those viruses for which it is known that foreign sequence is tolerated as part of the replicating viral genome. Included in these examples are tobacco mosaic virus (Dawson, W. O., et al (1989), *Virology*, 172: 285-292), tobacco etch virus (Dolja, V. V., et al (1992), *Proc. Natl. Acad. Sci. USA*, 89: 10208-10212), tobacco rattle virus (Ziegler-Graff, V., et al (1991), *Virology*, 182: 145-155), tomato bushy stunt virus (Scholthof, H. B., et al (1993), *Mol. Plant-Microbe*

Interact., 6: 309-322), brome mosaic virus (Mori, M., et al (1993), *J. Gen. Virol.*, 74: 1255-1260), cauliflower mosaic virus (Futterer, J. and Hohn, T. (1991), *EMBO J.*, 10: 3887-3896), african cassava mosaic virus (Ward, A., et al (1988), *EMBO J.*, 7: 1583-1587), tomato golden mosaic virus. Preferred viruses for use in the present invention may be RNA viruses.

A foreign targeting sequence may be included in the construct as a substitution for a viral gene or sequence that is not required for replication or as an additional sequence added to the viral genome. A foreign sequence may be included in the amplicon as an intact open reading frame and so that it is transcribed as a subgenomic RNA. However, a foreign sequence may be included anywhere in the viral cDNA irrespective of the location of subgenomic promoter.

A sequence foreign to the viral nucleic acid and homologous or similar to a target gene of interest, may be included in a sense or anti-sense orientation in the amplicon.

In using anti-sense genes or partial gene sequences to down-regulate gene expression, a nucleotide sequence is placed under the control of a promoter in a "reverse orientation" such that transcription yields RNA which is complementary to normal mRNA transcribed from the "sense" strand of the target gene. See, for example, Rothstein et al, 1987; Smith et al, (1988) *Nature* 334, 724-726; Zhang et al, (1992) *The Plant Cell* 4, 1575-1588, English

et al., (1996) *The Plant Cell* 8, 179-188. Antisense technology is also reviewed in Bourque, (1995), *Plant Science* 105, 125-149, and Flavell, (1994) *PNAS USA* 91, 3490-3496.

5 An alternative is to use a copy of all or part of the target gene inserted in sense, that is the same, orientation as the target gene, to achieve reduction in expression of the target gene by co-suppression. See, for example, van der Krol et al., (1990) *The Plant Cell*
10 2, 291-299; Napoli et al., (1990) *The Plant Cell* 2, 279-289; Zhang et al., (1992) *The Plant Cell* 4, 1575-1588, and US-A-5,231,020.

 The complete sequence corresponding to the coding sequence (in reverse orientation for anti-sense) need not
15 be used. For example fragments of sufficient length may be used. It is a routine matter for the person skilled in the art to screen fragments of various sizes and from various parts of the coding sequence to optimise the level of anti-sense inhibition. It may be advantageous
20 to include the initiating methionine ATG codon, and perhaps one or more nucleotides upstream of the initiating codon. A further possibility is to target a conserved sequence of a gene, e.g. a sequence that is characteristic of one or more genes in one or more
25 pathogens against which resistance is desired, such as a regulatory sequence.

 A foreign sequence may be 500 nucleotides or less, possibly about 400 nucleotides, about 300 nucleotides,

about 200 nucleotides, or about 100 nucleotides. It may be possible to use oligonucleotides of much shorter lengths, 14-23 nucleotides, although longer fragments, and generally even longer than 500 nucleotides are
5 preferable where possible.

It may be preferable that there is complete sequence identity in the targeting (e.g. foreign) sequence in the amplicon and the target sequence in the plant, though total complementarity or similarity of
10 sequence is not essential. One or more nucleotides may differ in the targeting sequence from the target gene. Thus, a targeting sequence employed in a construct in accordance with the present invention may be a wild-type sequence (e.g. gene) selected from those available, or a
15 mutant, derivative, variant or allele, by way of insertion, addition, deletion or substitution of one or more nucleotides, of such a sequence. A foreign sequence need not include an open reading frame or specify an RNA that would be translatable. As noted, a foreign sequence
20 may be inserted into the amplicon construct in either orientation, for sense or anti-sense regulation. It may be preferred for there to be sufficient homology for the respective anti-sense and sense RNA molecules to hybridise. There may be gaps even where there is about 5%,
25 10%, 15% or 20% or more mismatch between the targeting, e.g. foreign, sequence in the amplicon and the target gene.

In embodiments of the present invention which have

been experimentally exemplified as described below for illustrative and non-limiting purposes only, the foreign sequence in the amplicon that determined the target of gene silencing was the uidA reporter gene (Jefferson, R. A., et al (1986), *Proc. Natl. Acad. Sci. USA*, 83: 8447-8451) or the gene encoding the jellyfish green fluorescent protein (Chalfie et al. (1994) *Science* 263: 802-805). Other disclosed example genes include tomato DWARF and arabidopsis phytoene desaturase. However, any other gene of plant, animal, fungal, bacterial or viral origin may be a target of amplicon gs provided that the corresponding foreign sequence is incorporated into the amplicon construct. Particularly suitable as targets are genes that have already been shown to be suppressible by gs in the literature. These may include, for example, chalcone synthase of petunia or polygalacturonase of tomato (Jorgensen, R. A. (1995), *Science*, 268: 686-691, Hamilton, A. J., et al (1995), *Current Topics In Microbiology and Immunology*, 197: 77-89).

One or more targeting sequences may be included in the construct, to provide for suppression of one or more genes, e.g. when down-regulation of more than one gene is required at the same time, or for conferring resistance to more than one pathogen. A targeting sequence with sufficient homology to more than one target sequence may be used in suppressing more than one gene.

An additional optional feature of a construct used in accordance with the present invention is a

transcriptional terminator. The transcriptional terminator from nopaline synthase gene of agrobacterium tumefaciens (Depicker, A., et al (1982), *J. Mol. Appl. Genet.*, 1: 561-573) may be used, and is experimentally exemplified below. Other suitable transcriptional terminators include but are not restricted to those from soybean actin, ribulose biphosphate carboxylase of *Nicotiana plumbaginifolia* (Poulson, C., et al (1986), *Mol. Gen. Genet.*, 205: 193-200) and alpha amylase of wheat (Baulcombe, D. C., et al (1987), *Mol. Gen. Genet.*, 209: 33-40). A transcriptional terminator sequence foreign to the virus may not be included in a construct of the invention in particular when the viral sequences included in the construct include one or more transcriptional terminator sequences.

Those skilled in the art are well able to construct vectors and design protocols for recombinant gene expression. For further details see, for example, *Molecular Cloning: a Laboratory Manual*: 2nd edition, Sambrook et al, 1989, Cold Spring Harbor Laboratory Press.

Many known techniques and protocols for manipulation of nucleic acid, for example in preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in detail in *Protocols in Molecular Biology*, Second Edition, Ausubel et al. eds., John Wiley & Sons, 1992.

Specific procedures and vectors previously used with wide success upon plants are described by Bevan, Nucl. Acids Res. (1984) 12, 8711-8721), and Guerineau and Mullineaux, (1993) Plant transformation and expression
5 vectors. In: Plant Molecular Biology Labfax (Croy RRD ed) Oxford, BIOS Scientific Publishers, pp 121-148.

For introduction into a plant cell, the nucleic acid construct may be in the form of a recombinant vector, for example an *Agrobacterium* binary vector.
10 Microbial, particularly bacterial and especially *Agrobacterium*, host cells containing a construct according to the invention or a vector which includes such a construct, particularly a binary vector suitable for stable transformation of a plant cell, are also
15 provided by the present invention.

Nucleic acid molecules, constructs and vectors according to the present invention may be provided isolated and/or purified (i.e. from their natural environment), in substantially pure or homogeneous form,
20 or free or substantially free of other nucleic acid. Nucleic acid according to the present invention may be wholly or partially synthetic. The term "isolate" encompasses all these possibilities.

An aspect of the present invention is the use of a
25 construct or vector according to the invention in the production of a transgenic plant.

A further aspect provides a method including introducing the construct or vector into a plant cell

such that the construct is stably incorporated into the genome of the cell.

Any appropriate method of plant transformation may be used to generate plant cells containing a construct within the genome in accordance with the present invention. Following transformation, plants may be regenerated from transformed plant cells and tissue.

Successfully transformed cells and/or plants, i.e. with the construct incorporated into their genome, may be selected following introduction of the nucleic acid into plant cells, optionally followed by regeneration into a plant, e.g. using one or more marker genes such as antibiotic resistance. Selectable genetic markers may be used consisting of chimaeric genes that confer selectable phenotypes such as resistance to antibiotics such as kanamycin, hygromycin, phosphinotricin, chlorsulfuron, methotrexate, gentamycin, spectinomycin, imidazolinones and glyphosate.

When introducing a nucleic acid into a cell, certain considerations must be taken into account, well known to those skilled in the art. The nucleic acid to be inserted should be assembled within a construct which contains effective regulatory elements which will drive transcription. There must be available a method of transporting the construct into the cell. Once the construct is within the cell membrane, integration into the endogenous chromosomal material should occur.

Finally, as far as plants are concerned the target cell

type must be such that cells can be regenerated into whole plants.

Plants transformed with the DNA segment containing the sequence may be produced by standard techniques which are already known for the genetic manipulation of plants. DNA can be transformed into plant cells using any suitable technology, such as a disarmed Ti-plasmid vector carried by *Agrobacterium* exploiting its natural gene transfer ability (EP-A-270355, EP-A-0116718, NAR 12(22) 8711 - 87215 1984), particle or microprojectile bombardment (US 5100792, EP-A-444882, EP-A-434616) microinjection (WO 92/09696, WO 94/00583, EP 331083, EP 175966, Green et al. (1987) *Plant Tissue and Cell Culture*, Academic Press), electroporation (EP 290395, WO 8706614 Gelvin Debeyser - see attached) other forms of direct DNA uptake (DE 4005152, WO 9012096, US 4684611), liposome mediated DNA uptake (e.g. Freeman et al. *Plant Cell Physiol.* 29: 1353 (1984)), or the vortexing method (e.g. Kindle, *PNAS U.S.A.* 87: 1228 (1990d). Physical methods for the transformation of plant cells are reviewed in Oard, 1991, *Biotech. Adv.* 9: 1-11.

Agrobacterium transformation is widely used by those skilled in the art to transform dicotyledonous species. Recently, there has been substantial progress towards the routine production of stable, fertile transgenic plants in almost all economically relevant monocot plants (Toriyama, et al. (1988) *Bio/Technology* 6, 1072-1074; Zhang, et al. (1988) *Plant Cell Rep.* 7, 379-

- 384; Zhang, et al. (1988) *Theor Appl Genet* 76, 835-840; Shimamoto, et al. (1989) *Nature* 338, 274-276; Datta, et al. (1990) *Bio/Technology* 8, 736-740; Christou, et al. (1991) *Bio/Technology* 9, 957-962; Peng, et al. (1991)
- 5 International Rice Research Institute, Manila, Philippines 563-574; Cao, et al. (1992) *Plant Cell Rep.* 11, 585-591; Li, et al. (1993) *Plant Cell Rep.* 12, 250-255; Rathore, et al. (1993) *Plant Molecular Biology* 21, 871-884; Fromm, et al. (1990) *Bio/Technology* 8, 833-839;
- 10 Gordon-Kamm, et al. (1990) *Plant Cell* 2, 603-618; D'Halluin, et al. (1992) *Plant Cell* 4, 1495-1505; Walters, et al. (1992) *Plant Molecular Biology* 18, 189-200; Koziel, et al. (1993) *Biotechnology* 11, 194-200; Vasil, I. K. (1994) *Plant Molecular Biology* 25, 925-937;
- 15 Weeks, et al. (1993) *Plant Physiology* 102, 1077-1084; Somers, et al. (1992) *Bio/Technology* 10, 1589-1594; WO92/14828). In particular, *Agrobacterium* mediated transformation is now emerging also as an highly efficient transformation method in monocots (Hiei et al.
- 20 (1994) *The Plant Journal* 6, 271-282).

The generation of fertile transgenic plants has been achieved in the cereals rice, maize, wheat, oat, and barley (reviewed in Shimamoto, K. (1994) *Current Opinion in Biotechnology* 5, 158-162.; Vasil, et al. (1992)

25 *Bio/Technology* 10, 667-674; Vain et al., 1995, *Biotechnology Advances* 13 (4): 653-671; Vasil, 1996, *Nature Biotechnology* 14 page 702).

Microprojectile bombardment, electroporation and

direct DNA uptake are preferred where *Agrobacterium* is inefficient or ineffective. Alternatively, a combination of different techniques may be employed to enhance the efficiency of the transformation process, eg bombardment with *Agrobacterium* coated microparticles (EP-A-486234) or microprojectile bombardment to induce wounding followed by co-cultivation with *Agrobacterium* (EP-A-486233).

Following transformation, a plant may be regenerated, e.g. from single cells, callus tissue or leaf discs, as is standard in the art. Almost any plant can be entirely regenerated from cells, tissues and organs of the plant. Available techniques are reviewed in Vasil et al., *Cell Culture and Somatic Cell Genetics of Plants, Vol I, II and III, Laboratory Procedures and Their Applications*, Academic Press, 1984, and Weissbach and Weissbach, *Methods for Plant Molecular Biology*, Academic Press, 1989.

The particular choice of a transformation technology will be determined by its efficiency to transform certain plant species as well as the experience and preference of the person practising the invention with a particular methodology of choice. It will be apparent to the skilled person that the particular choice of a transformation system to introduce nucleic acid into plant cells is not essential to or a limitation of the invention, nor is the choice of technique for plant regeneration.

Also according to the invention there is provided a

plant cell having incorporated into its genome a DNA construct as disclosed. A further aspect of the present invention provides a method of making such a plant cell involving introduction of a vector including the
5 construct into a plant cell. Such introduction should be followed by recombination between the vector and the plant cell genome to introduce the sequence of nucleotides into the genome. RNA encoded by the introduced nucleic acid construct may then be transcribed
10 in the cell and descendants thereof, including cells in plants regenerated from transformed material. A gene stably incorporated into the genome of a plant is passed from generation to generation to descendants of the plant, so such descendants should show the desired
15 phenotype.

The present invention also provides a plant comprising a plant cell as disclosed.

A plant according to the present invention may be one which does not breed true in one or more properties.
20 Plant varieties may be excluded, particularly registrable plant varieties according to Plant Breeders' Rights. It is noted that a plant need not be considered a "plant variety" simply because it contains stably within its genome a transgene, introduced into a cell of the plant
25 or an ancestor thereof.

In addition to a plant, the present invention provides any clone of such a plant, seed, selfed or hybrid progeny and descendants, and any part of any of

these, such as cuttings, seed. The invention provides any plant propagule, that is any part which may be used in reproduction or propagation, sexual or asexual, including cuttings, seed and so on. Also encompassed by the invention is a plant which is a sexually or asexually propagated off-spring, clone or descendant of such a plant, or any part or propagule of said plant, off-spring, clone or descendant. Plant extracts and derivatives are also provided.

10 The present invention may particularly be applied in plants such as natural hosts of a plant virus, including any mentioned herein, though it is an advantage of embodiments of the present invention that viruses may be used for gene silencing in plants which are not their
15 natural hosts, as has been demonstrated experimentally and is described below. Indeed the present inventors have demonstrated that PVX, used in many of the Examples below, can replicate in monocots in addition to its natural hosts.

20 The present invention may be used in plants such as crop plants, including cereals and pulses, maize, wheat, potatoes, tapioca, rice, sorghum, millet, cassava, barley, pea and other root, tuber or seed crops. Important seed
25 crops are oil seed rape, sugar beet, maize, sunflower, soybean and sorghum. Horticultural plants to which the present invention may be applied may include lettuce, endive and vegetable brassicas including cabbage, broccoli and cauliflower, and carnations and geraniums.

The present invention may be applied to tobacco, cucurbits, carrot, strawberry, sunflower, tomato, pepper, chrysanthemum, poplar, eucalyptus and pine.

As noted, transcription from the construct in the genome of a plant cell yields a cytoplasmically-replicating RNA able to down-regulate expression of a target gene in the cell, which target gene may be of the plant, endogenous to the plant genome or a transgene, or of a pathogen such as a virus.

Thus, a further aspect of the present invention provides a method which includes causing or allowing transcription from a construct as disclosed within the genome of a plant cell.

A further aspect of the present invention provides a method of reducing or suppressing or lowering the level of expression of a gene of interest (or "target gene") in a plant cell, the method including causing or allowing transcription from a construct as disclosed.

Transcription produces RNA and is generally followed by replication of the transcribed RNA by virtue of the inclusion of the viral sequence. The construct may include the target gene sequence or a fragment thereof in a sense or anti-sense orientation, or a sequence with sufficient homology to the target gene sequence or a fragment thereof for the level of expression of the target gene to be reduced on production of the RNA.

As demonstrated in the Examples hereinafter, in certain embodiments the target gene may be remote from

the site of the amplicon replication, which may only be activated in certain cells (for instance because the amplicon is activated by an inducible promoter). In such cases the gs may be achieved through the demonstrated systemic or remote effect. In the light of the present work, it appears that this effect may be analagous to that disclosed in Voinnet & Baulcombe (1997) Nature 389: page 553, (although in that case the initiator of the systemic gs signal was not a cytoplasmically replicating construct). Thus the distal effect may be particularly effective when the amplicon is activated in a photosynthetic 'source' tissue, while the target tissue is present (either locally or systemically) in 'sink' tissue(s).

With respect to the level of expression of a gene of interest in a cell, the method will generally result in a decrease in the level of expression as compared with the level in the absence of the intervention, i.e. in comparison with equivalent wild-type cells, e.g. of plants of the same species. (Cells which are wild-type in respect of the level of expression of the gene of interest may of course not be wild-type in every respect.)

Where the target is a gene of a pathogen such as a virus, down-regulation of expression may provide an increase in resistance to the pathogen, particularly where the gene is required for, or is at least involved in, replication and/or infectivity (e.g. in an RNA-

dependent RNA polymerase in a virus such as cowpea mosaic virus (Sijen, T., et al (1996), *Plant Cell*, 8: 2277-2294)).

5 The present invention will now be illustrated and exemplified with reference to experimental results and the accompanying figures. Further aspects and embodiments of the present invention, and modifications of those disclosed herein, will be apparent to those skilled in the art. All documents mentioned anywhere
10 herein are incorporated by reference.

Figures

Figure 1 illustrates 35S expressed amplicon constructs that were transferred to *N. tabacum* cv Petite havana plants by *Agrobacterium* mediated transformation.
15 Each construct was inserted between the CaMV 35S promoter and the nos terminator sequence (Jones, J. D. G., et al (1992), *Transgenic Res.*, 1: 285-297). The various PVX ORFs are labelled: RdRp, RNA-dependent RNA polymerase; 25, 12, 8, the "triple gene block" genes (encoding
20 proteins of 25, 12, and 8kDa, respectively; CP, coat protein gene. The GUS sequence, shown as a hatched box, was inserted as an additional ORF in PVX/GUS/CP and was expressed from a duplicated CP subgenomic promoter, or as a direct replacement for the CP in PVX/GUS (referred to
25 as pGC3S in Chapman, S. N., et al (1992), *Plant J.*, 2: 549-557). PVX/ Δ REP/CP was essentially PVX/GUS but with a 1.7kb deletion within the RdRp ORF to prevent the

initiation of viral replication. Amplicon-mediated gs was observed in all plants expressing the PVX, PVX/GUS, or PVX/GUS/CP constructs.

Figure 2 shows a time course of symptom expression on manually inoculated amplicon and non-transformed plants. Twenty plants from each amplicon line were manually inoculated with 0.5 μ g of virion RNA. For each experiment, twenty non-transformed plants were also inoculated. The mean (\pm SE) percentage of plants showing symptoms at 5 to 14 days post inoculation are plotted. No symptoms were visible on any plant until 6 days post inoculation.

Figure 2(a): Plants were inoculated with TXS (PVX strain UK3). Solid line, non-transformed plants and PVX/ Δ REP/GUS plants (data pooled); dashed line, progeny of amplicon plants (PVX, PVX/GUS, and PVX/GUS/CP (data pooled)).

Figure 2(b): Plants were inoculated with NL1, NL2, CP2 and CP4 strains of PVX (data pooled). Solid line, non-transformed and PVX/ Δ REP/GUS plants; dashed line, amplicon plants expressing CP (PVX and PVX/GUS/CP (data pooled)); stippled line (which is partly obscured by the solid line), amplicon plants expressing PVX/GUS i.e. no CP present. Every plant showed symptoms of infection by 10 days post inoculation. A delay in the appearance of symptoms was observed with plants expressing a virus with CP.

Figure 3 shows the plasmid constructs used in the

transient GUS expression assay (particle bombardment)..

Figure 3(a): Schematic representation of the pUC-based plasmid pSLJ4D4 (not drawn to scale). The GUS sequence was inserted between the 35S promoter and ocs terminator sequence. The unique *Bam*HI site at the 3' end of the GUS ORF was used to insert each of the eight fragments of the PVX genome shown in Figure 3(b).

Figure 3(b): Schematic representation of the PVX genome. RdRp, RNA-dependent RNA polymerase; 25, 12, 8, the "triple gene block"; CP, coat protein gene. The bars below the diagram denote the regions of the genome separately cloned into the *Bam*HI site of pSLJ4D4. The direction of the arrow heads indicate the sense or antisense orientation of the fragments in the *Bam*HI site. The numbers adjacent to each bar denote the resulting plasmid clone. The size of the PVX fragments cloned were as follows: 436, 1.7kb; 437, 2.0kb; 438, 1.0kb; 439, 0.7kb; 441, 0.5kb. The PVX fragments used to produce constructs 437, 438, 439 and 441 had the same 3' terminus, mapping to nucleotide position 6391 in the PVX genome.

Figure 4 shows the GUS activity in PVX and PVX/ Δ REP/GUS amplicon leaf discs after particle bombardment with the constructs illustrated in Figure 3. The leaf discs were stained for GUS activity 1 day post bombardment, cleared in 70% ethanol, and the number of blue spots were counted. The histograms are of mean numbers (+/-SE) of GUS positive spots on PVX/ Δ REP/GUS

(Δ REP) (white bars (sense constructs)) and PVX (light shaded bars (sense constructs) and hatched bars (antisense constructs)) leaf discs. Each experiment contained 6 to 8 control leaf discs excised from non-transformed plants (black bar). Experimental data were standardised such that the mean for the control discs in each experiment was 100. Each construct was bombarded into 9 PVX plants and 6 PVX/ Δ REP/GUS plants (two leaf discs from each plant). For each construct the data have been pooled. T-tests showed that there was no significant difference in the number of spots with pSLJ4D4 on any plant (PVX, PVX/ Δ REP/GUS or non-transformed), or with any construct on the non-transformed control plants compared with the PVX/ Δ REP/GUS plants. The difference in the number of spots on the PVX plants with the GUS/PVX plasmids was significantly different ($P > 0.95$) from the number on the non-transformed plants with these plasmids.

Figure 5 shows a gel blot analysis of RNA extracted from amplicon X 35S/GUS plants. RNA was extracted from plants expressing a PVX/GUS/CP amplicon (Amp X NT), or expressing a 35S/GUS transgene (GUS X NT), or expressing both the PVX/GUS/CP amplicon and 35S/GUS transgene (GUS X Amp). The presence of the transgenes was confirmed by PCR analyses and Southern hybridisations. 5 micrograms of total RNA was electrophoresed on a 1% gel. The entire GUS sequence was used as a probe. The arrows indicate the GUS mRNA (GUS) and amplicon RNA (PVX) that

was encapsidated.

Figure 6 shows a gel blot analysis of RNA extracted from the amplicon X TGB plants. RNA was extracted from the plants expressing an amplicon, an amplicon plus 35S/TGB transgene, or the TGB transgene alone. Track 1 shows RNA extracted from a PVX/GUS plant (AMP). Tracks 2 to 5 show RNA extracted from 35S/TGB12+8 transgenic plants (TGB). Tracks 6 and 7 show RNA extracted from plants expressing a PVX/GUS amplicon and the 35S/TGB12+8 transgene (AMP X TGB). Tracks 8 and 9 show RNA extracted from plants expressing both the 35S/TGB12+8 transgene and a PVX amplicon (AMP X TGB). The probe used was the entire TGB 12+8 coding sequence. The arrows indicate the encapsidated viral RNA (PVX) or the TGB mRNA (TGB).

Figure 7 shows a gel blot analysis of RNA extracted from 35S/GUS scions. RNA was extracted 5 weeks post grafting from the 35S/GUS scions. The stock plants were a PVX/GUS amplicon (AMP) or a PVX/DREP/GUS amplicon (DREP) or non-transformed control plants (NT). 5 micrograms of total RNA was electrophoresed on a 1% gel. The entire GUS sequence was used as a probe. The GUS arrow indicates the GUS mRNA. The asterix points to a second larger GUS transcript that is produced in the 35S/GUS line (SA94084). A similar reduction in GUS mRNA was observed in two other 35S/GUS lines tested, that do not produce this second GUS transcript, when they were inoculated to GUS amplicon plants.

Figure 8 shows a gel blot analysis of RNA extracted

from Arabidopsis plants expressing a PVX/GUS/AtrbohD amplicon (Amplicon plants) or non-transformed control plants (C). The probe used was the 5' end of AtrbohD.

Examples

5 EXAMPLE 1

The demonstration that an amplicon transgene activates gs involved a series of constructs in which potato virus X (PVX) cDNAs were introduced as transgene constructs into the tobacco genome.

10 Figure 1 illustrates the PVX cDNA amplicon constructs that were assembled and introduced into *Nicotiana tabacum* cv Petite Havana by *Agrobacterium tumefaciens*-mediated transformation. One construct had the PVX_{UK3} genome intact ("PVX"; Figure 1). The other
15 constructs were also based on the PVX_{UK3} genome but were modified to include the β -glucuronidase (GUS) gene inserted either as an additional open reading frame (ORF) in the viral genome ("PVX/GUS/CP"; Figure 1) or as a replacement for the coat protein ("PVX/GUS"; Figure 1).

20 Both the PVX/GUS/CP and the PVX/GUS construct specified viral genomes that would not cause systemic infection if their RNA was inoculated to non transformed plants. In the PVX/GUS/CP construct there was a mutation in the PVX ORF 2 that encodes a protein required for
25 movement of PVX out of the inoculated cell. The virus specified by the PVX/GUS construct would also remain confined to the inoculated cell because the infected

cells would not produce coat protein.

A further amplicon construct pA209 that was tested in some of our experiments specified a defective PVX ORF3. As a result of this mutation the viral genome produced from the amplicon DNA, like that with the ORF2 and CP mutations, was movement defective. Plants were also transformed with a defective PVX/GUS construct encoding a non-functional RNA-dependent RNA polymerase ("PVX/ Δ REP/GUS"; Figure 1). The transgene-derived RNA from this construct would not initiate the viral replication cycle. Other control plants expressed GUS directly from the 35S promoter ("35S/GUS").

The amplicon lines selected for further detailed analysis were those in which there was a single transgene insert. These plant lines (5 to 7 independent lines for each construct) were identified by DNA gel blot analysis of the primary transformants (T_0 plants) and by the segregation ratios for GUS and/or the *nptII* gene in the T_1 self progeny (T_1 plants). However, the single locus nature of the inserts is not crucial: lines with multiple inserts behaved in the same way as those with single inserts.

It was concluded that the RNA transcribed from the amplicon constructs was replicated in the cells of the various transgenic lines because there was GUS activity in the lines carrying the PVX/GUS pA209 and PVX/GUS/CP constructs (Table I) and PVX particles in the lines in which the CP ORF was intact (PVX and PVX/GUS/CP).

Production of GUS or CP was not due to direct translation of these proteins from the transgene-derived RNA because there was no GUS in the plants carrying the PVX/ Δ REP/GUS construct.

5 The evidence that replication of the amplicon RNA in the transgenic plants had activated gs was from the analysis of amplicon-mediated gene expression and because there was resistance against PVX and suppression of transiently expressed DNA in the amplicon lines. The following paragraphs discuss these points in greater
10 detail.

 The first indication that expression of the wild type and derivative PVX RNAs was suppressed in the amplicon plants was the unexpected observation that these
15 plants were completely free of viral symptoms. The initial indication that gs was active in the amplicon lines was from an analysis of the levels of GUS activity in the plants. The levels of GUS activity in the amplicon plants were no higher and were as variable as
20 the levels in the 35S GUS lines (Table I). In the absence of gs it would be expected that the levels of GUS activity would be higher than in the 35S/GUS lines because of the amplification of the amplicon transcript by the replication process. The involvement of gs in the
25 phenotype of the amplicon lines was also supported by histochemical staining which revealed that GUS activity was not detected in many of the cells of these plants. The absence of GUS was attributed to gs.

The GUS producing cells were evident as spots of histochemical staining in every plant expressing the PVX/GUS/CP or PVX/GUS transgene and in all organs of these plants including true leaves, stems, cotyledons, and roots. The spotted pattern of GUS activity was not observed in the plants expressing the 35S/GUS transgene. There are a number of possible explanations for the spotted appearance of the amplicon leaves after histochemical staining for GUS. In principle the spots could develop because the amplicon mediated gs is somatically unstable. However we consider that somatic instability is unlikely for several reasons: the regions of GUS production did not correspond to developmental sectors in the plant; the levels of amplicon RNA were not as high as would be expected if the gs was not active in all leaves of the plant and there was resistance against PVX even in regions of the amplicon leaves that stained blue for GUS. An alternative explanation is that the gs and associated homology dependent resistance is somatically stable in the amplicon plants but that the replicable amplicon RNA occasionally overcomes the effects of this gs. Resistance breaking, manifest as spots of GUS activity, would be more obvious in the GUS amplicon lines than in the PVX lines manually inoculated with PVX.GUS because the *in planta* transcription of the amplicon would generate a higher inoculation pressure than with a manually inoculated virus.)

The characteristic attributes of virus resistance

by gs in transgenic plants are that it is specific to viruses that are very similar to the transgene at the nucleotide level and that it is often associated with low levels of the transgene RNA. Virus resistance with these characteristics is referred to as homology-dependent resistance.

To determine whether the amplicon lines (Figure 1) exhibited homology-dependent resistance the amplicon plants of the T₁ generation were inoculated with PVX_{UK3} which is the strain of PVX used as the original source of cDNA for the amplicon constructs.

Symptoms of PVX infection developed in 25% of the plants at the same time as in the plants expressing the PVX/ Δ REP/GUS transgene or in the non-transformed plants. The remaining 75% of the amplicon plants failed to develop symptoms by 14 days post inoculation (all of the control plants showed symptoms within 9 days post inoculation) (Figure 2).

This 3:1 segregation ratio of the resistance trait indicated that PVX resistance was expressed in most, if not all, of the transformed progeny of the amplicon lines. The data shown in Figure 2 are combined from several independent experiments and are particularly notable for the lack of variation between plants and between lines generated with each construct.

In contrast, there was little or no resistance against the PVX_{CP} and PVX_{NL} strains. As the nucleotide sequence of PVX_{CP} is 78% similar to the sequence of PVX_{UK3}

(30) it is concluded that the resistance in the amplicon lines is highly strain-specific.

To further analyse the strain-specific resistance a PVX.GFP construct was inoculated to non-transformed plants, to two PVX/GUS, two PVX/GUS/CP and two PVX/ Δ REP/CP lines. PVX.GFP is a PVX_{UK3} vector construct carrying the green fluorescent protein (GFP) reporter gene.

On the inoculated leaves of the non-transformed and PVX/ Δ REP/GUS plants there were many large spots of green fluorescence at 7 days post inoculation illustrating infection of PVX.GFP on these plants. In contrast, there were no fluorescent regions on the leaves of the amplicon plants.

Similarly, PVX.GUS (the PVX_{UK3} vector expressing the GUS reporter gene) produced large GUS-positive foci of infection on the PVX/ Δ REP/GUS and non-transformed plants, but not on PVX plants. Infrequently, and after prolonged histochemical staining, there were 3 to 6 small spots of GUS activity visible on the leaves of PVX plants inoculated with PVX.GUS.

Based on these reporter gene data it was concluded that the absence of symptoms following inoculation of the amplicon plants with PVX_{UK3} was due to resistance to initial infection rather than tolerance of PVX.

The conclusion that there was PVX resistance in the initially inoculated cell was confirmed by inoculation of PVX to protoplasts prepared from the transgenic plants.

The inocula for these protoplast assays were *in vitro* synthesised transcripts of either pTXS or pCP2 which are full length cDNA clones of PVX_{UK3} and PVX_{CP2}, respectively.

The protoplasts were prepared from plants
5 expressing each of the amplicon constructs or from non-transformed control plants. The protoplasts were inoculated with *in vitro* synthesised transcripts of pTXS, the cDNA clone of PVX_{UK3} (T) or pCP2, the cDNA clone of PVX_{CP2} (C). RNA was extracted from 50,000 protoplasts 24
10 hours post inoculation and electrophoresed on a 1% (w/v) agarose-formaldehyde gel, blotted onto nylon membrane, and hybridised to an RNA probe specific for the 3' end of PVX_{UK3} (T), or PVX_{UK3} (C). Autoradiographs were exposed for 1 hour.

15 The RNA gel blot analysis of the protoplast extracts revealed that the genomic and subgenomic RNAs of both strains accumulated at high levels in the protoplasts of the non-transformed or the PVX/ Δ REP/GUS lines. The PVX_{CP2} RNA accumulated to the same extent in
20 protoplasts of the amplicon and non-transformed lines. In contrast, the protoplasts of the amplicon lines displayed extreme resistance to PVX_{UK3}.

An important observation we made is that there is a low level of amplicon RNA in the mock inoculated cells of
25 the PVX or the PVX/GUS/CP lines. In the cells of the PVX/GUS the amplicon transcripts were barely detectable even after long exposure of the autoradiograph. These observations confirm that the PVX resistance in the

amplicon line was similar to homology-dependent resistance in that it was associated with low level accumulation of the transgene transcript.

EXAMPLE 2

5 Virus resistance, as shown in Example 1 above, could be considered as trans-acting gs of viral genes. To demonstrate that the amplicons could also mediate trans-acting gs of nuclear genes a transient assay was performed.

10 The constructs used in the transient assay were based on a plasmid expressing GUS directly from the 35S promoter (pSLJ4D4; Figure 3). Regions of the PVX_{UK3} cDNA clone were inserted on the 3' side of the GUS ORF. Sequences from either the 5' or 3' region of the viral
15 genome were inserted into pSLJ4D4 in both sense and antisense orientations (Figure 3). Each plasmid construct was coated onto gold particles and electrostatically bombarded into the leaves of plants expressing the PVX or PVX/ Δ REP/GUS transgene or non-
20 transformed plants.

 Histochemical staining for GUS activity of the bombarded leaves revealed that there was substantial and statistically significant suppression of GUS expression in the PVX plants, but only when the plasmid constructs
25 contained a region of the PVX genome (Figure 4). Suppression was manifested as a reduced number of blue spots on the PVX leaves compared with the number on the

non-transformed control plants (Figure 4). Based on the number of blue spots in this transient assay, the GUS expression in the PVX plants was 50 to 90% less than that observed in the non-transformed plants.

5 Expression of GUS from the plasmids with the PVX sequences transcribed in the antisense orientation was suppressed at least as well as from those where the PVX sequence was transcribed as sense RNA.

10 No suppression of GUS expression in the transient assay was ever observed in the plants with the PVX/ Δ REP/GUS transgene (Figure 4).

15 There was no suppression of GUS expression from pSLJ4D4 which has promoter homology with the amplicon transgene (Figure 4). It is likely therefore that the suppression of GUS expression from the GUS/PVX chimeric constructs involved a post-transcriptional mechanism in the amplicon plants. This mechanism resembles post-transcriptional gs in that it targets viral RNAs and RNAs transcribed from transiently expressed nuclear genes.

20 Presumably the PVX sequence at the 3' end of the GUS/PVX transcript from the transiently expressed plasmid was the target of a mechanism of post-transcriptional gs on the amplicon plants expressing the PVX transgene.

EXAMPLE 3

25 It was not possible to use the biolistic transient assay of gene silencing in the lines with PVX/GUS and PVX/GUS/CP amplicons because the bombardment procedure

led to high background levels of GUS. In order to analyse gene silencing in these lines an alternative transient assay of gs was performed.

5 The 35S/GUS sequence from pSLJ4D4 was transferred to a binary vector. *Agrobacterium tumefaciens* cultures carrying this construct were infiltrated into leaves of the amplicon plants and GUS expression ascertained by histochemistry staining. Expression of GUS was suppressed in both the PVX/GUS and the PVX/GUS/CP plants but not the PVX plants. Preliminary data suggest that the PVX/GUS amplicon is a better silencer of GUS than the PVX/GUS/CP construct.

15 In similar experiments the 35S/GUS/PVX sequence from plasmid 437 was transferred to a binary vector and introduced into the amplicon plants via *Agrobacterium* infiltration. Histochemical analysis revealed that GUS expression from the 437 construct was suppressed in PVX plants PVX/GUS plants.

20 The reproducibility of the amplicon gs is indicated by the above tests in which all amplicon lines tested exhibited a similar phenotype. A Table is presented (Table I) to show the number of lines used for each test.

Table I

	ANALYSIS	NO. OF LINES TESTED
25	Virus resistance	24
	Bombardment	5
	<i>Agrobacterium</i> Infiltration	8

EXAMPLE 4

In a third assay of amplicon gs the amplicon lines (PVX/GUS, PVX/GUS/CP & PVX/ Δ REP/GUS) were crossed to lines carrying a 35S/GUS transgene. RNA gel blot
5 analysis showed a suppression of GUS mRNA in plants carrying both the amplicon and 35S/GUS transgenes compared to plants carrying the 35S/GUS transgene alone (Figure 5).

These data indicate that the amplicon can mediate
10 gs of a nuclear gene with homology to the amplicon construct.

Reciprocal crosses were made between the *Nicotiana tabacum* cv. Petite amplicon plants (PVX or PVX/GUS or PVX/ Δ REP/GUS) or non-transformed control plants) and
15 35S/TGB12+8 transgenic plants. The 35S/TGB12+8 plants express the PVX 12kDa and 8kDa triple gene block proteins. These plants show an extreme dwarf phenotype and have chlorotic and necrotic leaves.

All plants expressing both the PVX amplicon and the
20 35S/TGB12+8 transgene were wild-type in appearance, that is they lost the chlorosis and necrosis and were of normal size. All plants expressing both the PVX/ Δ REP/GUS amplicon and the 35S/TGB12+8 transgene showed the TGB phenotype, consistent with the hypothesis
25 that replication of the amplicon is required to trigger the gene silencing mechanism. RNA gel blot analysis

confirmed that the TGB mRNA was reduced in the plants silenced by the amplicon (Figure 6).

EXAMPLE 5

In a fourth assay of amplicon gs, the 35S/GUS transgenic plants were grafted onto amplicon plants (PVX/GUS/CP, PVX/GUS, PVX, or PVX/ Δ REP/GUS) or non-transformed (NT) control plants. Gel blot analysis on RNA extracted from the 35S/GUS scions showed that the GUS mRNA was reduced when the stock plant expressed a replicating PVX/GUS or PVX/GUS/CP amplicon (Figure 7). These data indicate that the amplicon produces a systemic signal of gs which can suppress expression of a transgene with homology to the amplicon construct.

This indicates that the systemic or remote amplicon induced gs may be observed in those embodiments wherein not all cells in a plant are transcribing the amplicon encoding nucleic acid, for instance because it has not been introduced throughout the plant, or because transcription is under the control of an inducible or tissue- or developmentally- regulated promoters.

EXAMPLE 6

In a further assay of amplicon gs, part of the tomato dwarf gene was inserted into a PVX/GUS amplicon. The resulting construct, PVX/GUS/DWARF, was introduced into tomato plants using *A. tumefaciens* -mediated transformation. Histochemical staining revealed spots of

GUS activity on the transformed plants, similar to the pattern of GUS staining observed on tobacco plants expressing a PVX/GUS or PVX/GUS/CP amplicon. This observation suggested that the PVX/GUS/DWARF amplicon was expressed and viral replication had induced gs in the tomato plants. All of the plants expressing the PVX/GUS/DWARF amplicon showed reduced plant height compared to non-transformed control plants taken through the tissue culture procedure at the same time (plants were assessed 3-4 weeks after being transferred to rooting media, and were grown at 24°C with 16 hours of illumination. These data indicate that the amplicon can activate reproducible gs in tomato and can suppress expression of an endogenous gene.

EXAMPLE 7

Amplicon gs does not rely on use of the 35S promoter of CaMV. We have repeated the transformations described above with constructs that are similar to the 35S promoter constructs shown in Figure 1 except that they have the nos promoter of *Agrobacterium tumefaciens*.

Analyses performed on the primary transformants revealed that the plants showed little or no GUS activity (nos/PVX/GUS and nos/PVX/GUS/CP plants), but contained virions (nos/PVX and nos PVX/GUS/CP plants), similar to the observations made with the 35S expressed amplicons.

The nos amplicon plants were infiltrated with *Agrobacterium* cultures carrying a 35S/GUS plasmid and

infiltrated leaves stained for GUS activity 2 days post infiltration. Panels of GUS activity were observed on leaves from non-transformed control plants and from leaves expressing a nos/PVX or nos/PVX/ Δ REP/GUS amplicon.

5 GUS activity from the transiently expressed plasmid was not observed in leaves expressing a nos/PVX/GUS or nos/PVX/GUS/CP amplicon.

These data indicate that the nos amplicons act in the same way as the 35S amplicons in that they can
10 mediate reproducible gs.

EXAMPLE 8

PVX amplicons may be used in a range of plants including those that are not natural hosts of PVX.

To demonstrate this host range aspect of the
15 amplicon phenotype, a series of amplicon constructs were assembled that are similar to those shown in Figure 1 with the exception that the GUS sequence was replaced with cDNA encoding the jellyfish green fluorescent protein (GFP). These GFP amplicon constructs were
20 essentially identical to the PVX.GFP vector constructs that have been described previously (in Baulcombe et al. (1995), *Plant* 7: 1045-1053). These constructs were transformed into *Arabidopsis thaliana* which is not a host for PVX.

25 The transgenic plants contained particles (virions) of PVX.GFP indicating that the RNA transcript of the amplicon had undergone replication in *Arabidopsis*.

Seedlings transformed with a PVX/GFP/CP amplicon showed green fluorescence from the GFP reported gene at the tips of the very first true leaves only. GFP was not detected in older plants.

5 Additionally, a plant was inoculated with sap extracted from an *Arabidopsis* plant transformed with a PVX/GFP/CP amplicon. A leaf was illuminated under UV light and showed spots of GFP activity indicating the presence of PVX/GFP/CP virions in the *Arabidopsis* sap
10 extract.

 The Progeny of these transformed *Arabidopsis* also contained particles of PVX.GFP indicating that the amplicon was integrated into the nuclear genome and inherited. However, despite the presence of PVX.GFP
15 particles there was no detectable GFP in these lines.

 Thus, the phenotype of these *Arabidopsis* lines parallels the phenotype of the transgenic tobacco plants described above: there is virus replication but there is suppression of the reporter gene (GFP in this case) that
20 is part of the amplicon construct. These features are strong indicators that there is gs due to the presence of the amplicon in the genome of *Arabidopsis*.

 In a further experiment, *Arabidopsis* plants were transformed with a 35S/GFP transgene. These plants
25 were cross pollinated with plants expressing a PVX/GFP/CP amplicon or with non-transformed (NT) control plants. Both the parental 35S/GFP transgenic plants and the progeny from the cross 35S/GFP X NT showed uniform GFP

expression. However, every plant expressing both the PVX/GFP/CP amplicon and the 35S/GFP transgene showed no GFP activity. The 35S/GFP transgenic plants were also transformed again with a PVX/GFP amplicon. The
5 resulting transgenic plants showed no GFP activity despite carrying the 35S/GFP transgene. These observations indicate that the amplicon can mediate reproducible gs in *Arabidopsis*.

EXAMPLE 9

10 To demonstrate that amplicons can mediate reproducible gs of endogenes in *Arabidopsis*, part of the phytoene desaturase (PDS), ALBINO3, and *A. thaliana* respiratory burst oxidase homologue D (AtrbohD) endogenes were separately cloned into a PVX amplicon and were
15 transformed into *Arabidopsis*. Every transformant expressing a PVX/PDS or PVX/ALBINO amplicon showed white spots on the leaves, and AtrbohD mRNA levels were suppressed in every transformant expressing the PVX/AtrbohD amplicon (Figure 8). These data
20 demonstrate that the amplicon can mediate reproducible gs of endogenes in plants, even those which are not natural hosts for PVX. Thus amplicon-mediated gs may be used with endogenous genes including those responsible or associated with traits such as ripening, pollen
25 formation, lignin biosynthesis, flower pigment production, regulatory pathways controlling development, environmental responses (e.g. growth habit or disease

resistance), toxin production etc.

CONCLUSIONS FROM EXAMPLES ABOVE

The experimental evidence described above provides indications that amplicon constructs can cause gs of multiple targets. In the 35S/PVX/GUS lines there is
5 homology-dependent resistance against both PVX and suppression of GUS in the transient assay.

Amplicon constructs may be useful activators of gs, even with further elements deleted from the constructs
10 shown in Figure 1. Thus constructs based on those disclosed above, but comprising only a minimal amplicon, wherein all sequence from the viral construct that does not encode proteins required for replication or that does not include cis-acting elements required for replication,
15 has been eliminated. It should also be noted that gs apparently does not require the target sequence to be attached to a viral promoter because the 5' region of the viral genome which is not in a viral subgenomic RNA is a target of gene silencing.

20 EXAMPLE 10

COMPARISON WITH THE APPROACH SUGGESTED BY BIOSOURCE TECHNOLOGIES IN WO95/34668

The work of Biosource Technologies Inc (20,21) can be considered as following from the potyviral transgenes
25 work (22) mentioned above, although the sequence similarity between the virus and the nuclear genome

involves an endogenous nuclear gene rather than a transgene and there is no characterization of the virus induced gs on virus accumulation..

The paper (21 - Kumagai et al.) and the associated
5 patent application (20) describe two types of examples involving TMV vectors.

In one of these the cDNA sequence inserted into the vector was of phytoene desaturase mRNA and there is virus-induced silencing of the endogenous phytoene
10 desaturase gene. A second example involves phytoene synthase cDNA added into the TMV vectors. In this example there is no silencing of the endogenous gene. In fact there was over-expression of the phytoene synthase. The differences between these two examples are further
15 evidence that virus induced gene silencing is not necessarily a reproducible phenomenon.

An additional important point about variation in virus-induced gene silencing has been demonstrated by our further experimental analysis of the Biosource documents.

20 These analyses have revealed that virus induced gene silencing varies between plant species. We have produced a virus vector construct similar to their TMV/phytoene desaturase construct. The only major difference between our construct and the specific
25 Biosource experiments is that the virus vector is potato virus X rather than TMV. However, this difference is not likely to influence the outcome of the experiments because a PVX construct, like the TMV construct is able

to initiate silencing of the endogenous PDS gene when inoculated to *Nicotiana benthamiana*. This vector is also able to silence the PDS of *Nicotiana clevelandii*.

However, there is no silencing of the PDS gene of *Lycopersicon esculentum* even though this plant is a recognised host of PVX.

We introduced a fragment of the tomato phytoene desaturase (PDS) cDNA into the PVX vector and inoculated to *Nicotiana benthamiana*, *Nicotiana clevelandii* and *Lycopersicon esculentum* (tomato). The fragment of tomato PDS sequence inserted into the PVX vector was exactly the same as the fragment added to the TMV vector in the experiments of Kumagai et al. (21). When the PVX/PDS vector constructs were inoculated to the *Nicotiana* species, as described by Kumagai et al with the TMV constructs, there was photobleaching of the upper parts of the plant indicating that there had been gs of the endogenous PDS genes. However, when the same PVX/PDS constructs were inoculated to tomato, despite tomato being a good host of PVX and despite the identity of the viral PDS sequence and the endogenous sequence there was no photobleaching and therefore no gs of the tomato PDS.

The failure to get photobleaching could not be attributed to lack of infection: there were PVX symptoms on the infected plants and PVX/PDS RNA had accumulated to a high level.

In a similar experiment the cDNA (between the start codon at position 105 to the stop codon of the open

reading frame at position 1542) of glutamate semialdehyde
aminotransferase (GSA-AT2) of *Nicotiana tabacum* was
inserted into the PVX vector (Hofgen et al., (1994) *Proc.*
Natl. Acad. Sci. USA 91: 1726-1730). In this experiment
5 there was strong gs of the endogenous GSA-AT2 sequence
after inoculation to *N. benthamiana* and the infected
plants developed a yellow chlorosis. However after
inoculation to *N. tabacum* there was much weaker gs and
only a very mild chlorosis.

10 These results illustrate differences between the
amplicon gs and the virus-induced gs of the Biosource
documents. Amplicon gs does not rely on the ability of
the virus to move in the infected plant and may be used
even with plants that are not recognised hosts of the
15 virus used as a component of the amplicon construct.
Amplicon gs is useful even when the amplicon specifies a
defective construct that does not move from cell to cell
in the infected plant. In plant species in which the
amplicon transcripts replicate, our experiments show the
20 gs is consistent from plant to plant.

On the other hand, our experiments show that gs
induced by an inoculated virus is subject to plant to
plant variation associated with environmental effects on
virus spread. It is an effect that does not operate
25 equally well even in different hosts of the virus vector
and our experiments show it does not have the consistency
achieved using the present invention.

This influence of the host plant does not apply to

amplicon-mediated gene silencing in accordance with the present invention, which, as indicated above, is effective in plants that may or may not be a natural host for the viral component of the amplicon construct.

5 Materials and methods

Constructs and plant transformation

All of the viral cDNA constructs were inserted between the CaMV 35S promoter and the transcriptional terminator of the nopaline synthase (*nos*) gene and transferred into the binary vector pSLJ7291. Details of
10 each construct are given below.

An infectious PVX cDNA clone was inserted between the CaMV 35S promoter and the transcriptional terminator of the nopaline synthase (*nos*) gene to generate the
15 plasmid, pNCX. The 7.3kb *NarI* fragment encompassing the 35S promoter, the entire PVX genome, and terminator sequence was ligated with *ClaI* digested binary vector, pSLJ7291, to create the PVX transgene construct (Figure 1).

20 The 1.4kb *ApaI*-*XhoI* fragment from pNCX was replaced with the corresponding fragment from plasmid ΔG (7) to generate plasmid pA100. Plasmid pA100 is a pUC-based vector encoding the 35S promoter, PVX orfs 1,2,3,4, GUS and terminator sequence. A CP subgenomic promoter
25 sequence upstream of the GUS gene allowed GUS to be expressed as part of a subgenomic RNA (7). The 8.6kb *NarI* fragment from pA100 was ligated with *ClaI* digested

pSLJ7291, to create the PVX/GUS transgene construct (Figure 1).

A 1.7kb *Bgl*III fragment was deleted from PVX orf1 in plasmid pA100 to generate pA102. The 6.9kb *Nar*I fragment
5 from pA102 was ligated with *Cla*I digested pSLJ7291 to create the PVX/ Δ REP/GUS transgene construct (Figure 1).

The 1.4kb *Apa*I-*Xho*I fragment from pNCX was replaced with the corresponding fragment from plasmid pA Δ Apa/Apa-GUS (which is construct GC3 (7) containing a 324nt
10 deletion in orf2) to generate plasmid pA104. Plasmid pA104 is a pUC-based vector encoding the 35S promoter, PVX orf1, a mutated orf2, orfs 3 and 4, GUS, CP and terminator sequence. A duplicated CP subgenomic promoter sequence upstream of the GUS gene allowed GUS to be
15 expressed as part of a subgenomic RNA (7). The deletion in PVX orf2 disrupts cell-to-cell movement, but has little or no effect on viral accumulation in protoplasts (2). The 9.0kb *Nar*I fragment from pA104 was ligated with *Cla*I digested pSLJ7291, to create the PVX/GUS/CP
20 transgene construct (Figure 1).

The 35S/GUS construct (Figure 1) was generated by digestion of pSLJ4D4 (Figure 3) with *Eco*RII and *Hind*III and the 4.1kb fragment encoding the 35S promoter, GUS and terminator sequence was ligated with the similarly
25 digested binary vector, pSLJ7292, to generate the 35S/GUS transgene construct (Figure 1). Plasmid pSLJ7292 is identical to pSLJ7291 except for the orientation of the polylinker sequence.

Each construct was introduced into *N. tabacum* cv Petite havana by *A. tumefaciens* mediated leaf disc transformation (15) and plants were regenerated on medium containing kanamycin (300 µg/ml).

5 For the DWARF construct, 1kb of the 5' end of the tomato DWARF gene was inserted into the XhoI site of the 35S/PVX/GUS amplicon. The 9.6kb 35S/PVX/GUS/DWARF SstI fragment was ligated with SstI cut pSLJ7291. The construct was introduced into tomato cv Money Maker by *A.*
10 *tumefaciens* mediated leaf disc transformation (15) and plants were regenerated on medium containing kanamycin (300 micrograms/ml).

Other constructs (PVX/AtrbohD, PVX/ALBINO3, and PVX/PDS1) were constructed by introducing approximately
15 0.5-1.0kb of each target gene into the XhoI site of the 35S/PVX/GUS amplicon construct. Each construct was transferred into the binary vector pGREEN0029 (PVX/PDS and PVX/ALBINIO3) or pSLJ755 (PVX/AtrbohD) and introduced into *A. thaliana* plants by flower transformation.
20 Transgenic seedlings were selected after treatment with basta.

Analysis of GUS activity

Histochemical staining for GUS activity was
25 performed on T₀ and T₁ plants, as described (1). MUG assays on T₁ plants (5 weeks old) were performed as described (7). For each plant, leaf discs were excised from three different leaves and the sap extract pooled.

Preparation of PVX virions and virion RNA

The preparation of virions and virion RNA was as described previously (6).

*Transcription in vitro, preparation and electroporation
5 of protoplasts, and RNA gel blot analyses*

The plasmids pTXS and pCP2 were linearised with SpeI restriction enzyme and transcribed in vitro as described (7). The preparation and inoculation of protoplasts and RNA gel blot analysis were as described
10 (7).

Plasmid constructs for leaf disc bombardment

Fragments of the PVX genome (Figure 3) were generated by the polymerase chain reaction using oligonucleotide primers that contained a BamHI
15 restriction enzyme site in addition to the PVX sequence. The template DNA was pTXS (the full-length cDNA clone of PVX_{UK3}). The reactions were performed as described previously(7).

The DNA products were digested with BamHI and
20 ligated with BamHI digested pSLJ4D4 to generate the plasmids 437, 438, 439, and 441. The 1.7kb BglIII fragment from pTXS was ligated with BamHI digested pSLJ4D4 to generate the plasmid 436. The orientation of the PVX sequence in pSLJ4D4 was determined by restriction
25 enzyme analyses. The PVX fragments used for constructs 436, 437 and 438 were individually cloned in both sense

and antisense orientations (Figure 3).

Leaf disc bombardment

Leaf discs were excised from fully expanded leaves from 6 week old plants and placed onto Murashige and Skoog medium containing 3% sucrose and were immediately bombarded with gold particles coated with plasmid DNAs. Plasmid DNAs were prepared using a QIAGEN-tip 100 column (Qiagen Inc. Dorking, Surrey) and were precipitated onto 0.95 μ m diameter gold particles as described (25), but with a further 20-fold dilution of the preparation. Particle acceleration was performed using the ACCELL gene delivery system (Agracetus Inc.) under partial vacuum using a helium pressure of 10,000kPa. The leaf discs were incubated at RT in the dark for 24h and then stained for GUS activity.

REFERENCES

1. Angell, S.M. and Baulcombe, D.C. (1995) *Plant J.*, 7, 135-140.
2. Angell, et al. (1996) *Virology*, 215, 197-201.
- 20 3. Baulcombe, D.C. (1996) *Plant Molecular Biology*, 32, 79-88.
4. Baulcombe, D.C. and English, J.J. (1996) *Current Opinion In Biotechnology*, 7, 173-180.
5. Baulcombe et al. Gene silencing and virus resistance in transgenic plants. In: *Mechanisms and*
- 25

- applications of gene silencing, edited by Grierson, D., Lycett, G.W. and Tucker, G.A. Nottingham: Nottingham University Press, 1996, p. 127-138.
6. Baulcombe et al. (1984) *Plant Pathol.*, 33, 361-370.
 - 5 7. Chapman et al. (1992) *Plant J.*, 2, 549-557.
 8. Davies et al. (1993) *Virology*, 197, 166-175.
 9. Donson et al. (1994) *US-A-* 5,316,931.
 - 10 10. English et al. (1996) *Plant Cell*, 8, 179-188.
 11. Finnegan, J. and McElroy, D. (1994) *Bio/Technology*, 12, 883-888.
 12. Flavell, R.B. (1994) *Proc. Natl. Acad. Sci. USA*, 91, 3490-3496.
 13. Hobbs et al. (1990) *Plant Mol. Biol.*, 15, 851-864.
 14. Hobbs et al. (1993) *Plant Mol. Biol.*, 21, 17-26.
 - 15 15. Horsch et al. (1985) *Science*, 227, 1229-1231.
 16. Jorgensen, R.A. (1992) *AgBiotech News and Information*, 4, 265-273.
 17. Jorgensen, R.A. (1995) *Science*, 268, 686-691.
 18. Kaido et al. (1995) *J. Gen. Virol.*, 76, 2827-2833.
 - 20 19. Kooter et al. (1994) *Journal Of Cellular Biochemistry*, 115.
 20. Kumagai et al. (1995) WO95/34668.
 21. Kumagai et al. (1995) *Proc. Natl. Acad. Sci. USA*, 92, 1679-1683.
 - 25 22. Lindbo et al. (1993) *Plant Cell*, 5, 1749-1759.
 23. Matzke, M.A. and Matzke, A.J.M. (1995) *Trends In Genetics*, 11, 1-3.
 24. Matzke, M.A. and Matzke, A.J.M. (1995) *Plant*

Physiol., 107, 6679-685.

25. McCabe et al. (1988) *Bio/Technology*, 6, 923-926.
26. (deleted)
27. Mori et al. (1996) *Febs Lett.*, 1, 171-174.
- 5 28. Mueller et al. (1995) *Plant J.*, 7, 1001-1013.
29. Napoli et al. (1990) *Plant Cell*, 2, 279-289.
30. Santa Cruz, S. and Baulcombe, D.C. (1995) *J. Gen. Virol.*, 76, 2057-2061.
31. Suzuki et al. (1996) *Febs Lett.*, 379, 26-30.
- 10 32. Turpen, T.H. (1994) WO94/16089.
33. Turpen et al. (1996) WO96/12028.
34. Van Blokland et al. (1994) *Plant J.*, 6, 861-877.
35. van der Krol et al. (1990) *Plant Cell*, 2, 291-299.
36. Yamaya et al. (1988) *Mol. Gen. Genet.*, 215,
15 173-175.
37. Yamaya et al. (1988) *Mol. Gen. Genet.*, 211,
520-525.
- Baulcombe et al. (1987) *Mol. Gen. Genet.*, 209, 33-40.
- Baulcombe et al. (1995) *Plant J.*, 7, 1045-1053.
- 20 Bishop et al. (1996) *Plant Cell*, 8, 959-969.
- Dawson et al. (1989) *Virology*, 172, 285-292.
- Depicker et al. (1982) *J. Mol. Appl. Genet.*, 1, 561-573.
- Dolja et al. (1992) *Proc. Natl. Acad. Sci. USA*, 89,
10208-10212.
- 25 Futterer, J. and Hohn. T. (1991) *EMBO J.*, 10, 3887-3896.
- Hamilton et al. (1995) *Current Topics In Microbiology And Immunology*, 197, 77-89.

- Jefferson et al. (1986) *Proc. Natl. Acad. Sci. USA*, 83, 8447-8451.
- Jones et al. (1992) *Transgenic Res.*, 1, 285-297.
- Kavanagh et al. (1992) *Virology*, 189, 609-617.
- 5 Matzke, M.A. and Matzke, A.J.M. (1993) *Annu. Rev. Plant Physiol. Plant. Mol. Biol.*, 44, 53-76.
- Mori et al. (1993) *J. Gen. Virol.*, 74, 1255-1260.
- Park et al. (1996) *Plant J.*, 9, 183-194.
- Poulson et al. (1986) *Mol. Gen. Genet.*, 205, 193-200.
- 10 Sanders et al. (1987) *Nucleic Acids Res.*, 15, 1543-1558.
- Scholthof et al. (1993), *Mol. Plant-Microbe Interact.*, 6, 309-322.
- Sijen et al. (1996), *Plant Cell*, 8, 2277-2294.
- Torii et al. (1996) *Plant Cell*, 8, 735-746.
- 15 Vaucheret, H. (1993) *C. R. Acad. Sci. Paris*, 316, 1471-1483.
- Vaucheret, H. (1994) *C. R. Acad. Sci. Paris*, 317, 310-323.
- Walker, J.C. (1993) *Plant J.*, 3, 451-456.
- 20 Ward et al. (1988) *EMBO J.*, 7, 1583-1587.
- Ziegler-Graff et al. (1991) *Virology*, 182, 145-155.

CLAIMS

1 A DNA construct comprising a promoter operably linked to DNA which can be transcribed in a plant cell to an RNA transcript, which RNA transcript includes plant virus sequence which confers on the RNA transcript the ability to replicate in the cytoplasm of the plant cell.

2 A DNA construct as claimed in claim 1, wherein the plant virus is an RNA virus.

3 A DNA construct as claimed in claim 1, wherein the plant virus is a DNA virus.

4 A DNA construct as claimed in any one of the preceding claims wherein the plant virus is: potato virus X; tobacco mosaic virus; tobacco etch virus; tobacco rattle virus; tomato bushy stunt virus; bromo mosaic virus; cauliflower mosaic virus; african cassava mosaic virus; or tomato golden mosaic virus.

5 A DNA construct as claimed in any one of the preceding claims wherein the promoter is constitutive.

6 A DNA construct as claimed in any one of the claims 1 to 5 wherein the promoter is: developmentally regulated, inducible, or tissue specific.

7 A DNA construct as claimed in any one of the preceding claims wherein the promoter is selected from: CMV 35S; nos; GST-II-27 gene.

8 A DNA construct as claimed in any one of the preceding claims comprising a transcriptional terminator.

9 A DNA construct as claimed in any one of the preceding claims wherein the RNA transcript lacks all or part of the viral genome not required for replication in the cytoplasm.

10 A DNA construct as claimed in claim 9, wherein the RNA transcript cannot initiate systemic infection of a non-transformed plant by the plant virus from which the plant virus sequence is derived.

11 A DNA construct as claimed in claim 10, wherein the RNA transcript does not encode a coat protein of the plant virus from which the plant virus sequence is derived.

12 A DNA construct as claimed in any one of the preceding claims wherein the RNA transcript includes a targeting sequence which is capable of down-regulating expression of a target gene.

13 A DNA construct as claimed in claim 12 wherein the targeting sequence is, in a sense or anti-sense orientation, either:

(a) complementary to a sequence within the target gene,

or

(b) homologous to a sequence within the target gene.

14 A DNA construct as claimed in claim 13 wherein the homology between the targeting sequence and the sequence within the target gene is greater than: 80, 85, 90 or 95%.

15 A DNA construct as claimed in any one claims 12 to 14 wherein the targeting sequence targets at least the initiating ATG codon of the target gene.

16 A DNA construct as claimed in any one claims 12 to 15 wherein the targeting sequence targets a conserved sequence within a target gene group such as to down-regulate expression of one or more members of a target gene group.

17 A DNA construct as claimed in any one claims 12 to 16 comprising more than one targeting sequence.

18 A DNA construct as claimed in any one claims 12 to 17 wherein the targeting sequence is a sequence foreign to the plant virus sequence.

19 A DNA construct as claimed in claim 18 wherein the foreign sequence is substituted for a viral gene.

20 A DNA construct as claimed in claim 18 wherein the foreign sequence is additional to the viral genome.

21 A DNA construct as claimed in any one of claims 18 to 20 wherein foreign sequence is included in the construct as an intact open reading frame such that it is transcribed from the RNA transcript as a subgenomic RNA.

22 A DNA construct as claimed in any one of claims 18 to 21 wherein the foreign gene is: an endogenous plant gene, a transgene, or a gene from a pathogen.

23 A DNA construct as claimed in claim 22 wherein the endogenous plant gene is one associated with one or more of the following traits: ripening, pollen formation, lignin biosynthesis, flower pigment production, regulatory pathways controlling development, environmental responses, growth, disease resistance, toxin production.

24 A recombinant vector comprising a DNA construct of any one of the preceding claims.

25 A recombinant vector as claimed in claim 24 which

is suitable for stable transformation of a plant cell.

26 A recombinant vector as claimed in claim 25 which is an *Agrobacterium* binary vector.

27 Use of a DNA construct or recombinant vector of any one the preceding claims in the production of a transgenic plant.

28 A host cell containing a DNA construct or recombinant vector of any one of claims 1 to 26.

29 A host cell as claimed in claim 28 which is selected from: a microbial cell; a plant cell.

30 A host cell as claimed in claim 29 which is a plant cell having incorporated into its genome the DNA-construct of any one of claims 1 to 23.

31 A method of down-regulating expression of a target gene in a plant including the step of introducing a construct or vector of any one of claims 1 to 26 into a plant cell such that the construct is stably incorporated into the genome of the cell.

32 A method as claimed in claim 31 further comprising the step of regenerating a plant from the plant cell.

33 A plant comprising the plant cell of claim 30.

34 A plant as claimed in claim 33 which is a natural host of the plant virus from which the plant virus sequence of the construct is derived.

35 A plant as claimed in claim 33 or claim 34 which is; a cereal, a pulse, maize, wheat, potato, tapioca, rice, sorghum, millet, cassava, barley, pea, oil seed rape, sugar beet, maize, sunflower, soybean, sorghum, lettuce, endive, cabbage, onion, broccoli, cauliflower, carnation, geranium, canola, tobacco, cotton, cucurbits, carrot, strawberry, tomato, mango, peach, apple, pear, banana, melon, pepper, chrysanthemum, poplar, eucalyptus and pine.

36 A plant which is clone of the plant of any one claims 33 to 35.

37 A plant which is a descendant of the plant of any of claims 33 to 36

38 A plant propagule of the plant of any of claims 33 to 37.

39 A plant propagule as claimed in claim 38 which is a seed.

40 An extract or derivative of the plant or plant propagule of any one of claims 33 to 39.

41 A method of down-regulating expression of a target gene in a plant which includes causing or allowing transcription from a construct of any one of claims 1 to 23 within the genome of a plant cell of that plant.

42 A method as claimed in claim 41 wherein the site of the target gene in the plant is remote from cell in which the construct is transcribed.

43 A method of increasing the resistance of a plant to a pathogen comprising the method of claim 41 or claim 42.

44 A method as claimed in claim 43 where the target gene is involved in replication and/or infectivity of the pathogen.

45 A method as claimed in claim 43 or claim 44 wherein the pathogen is a virus.

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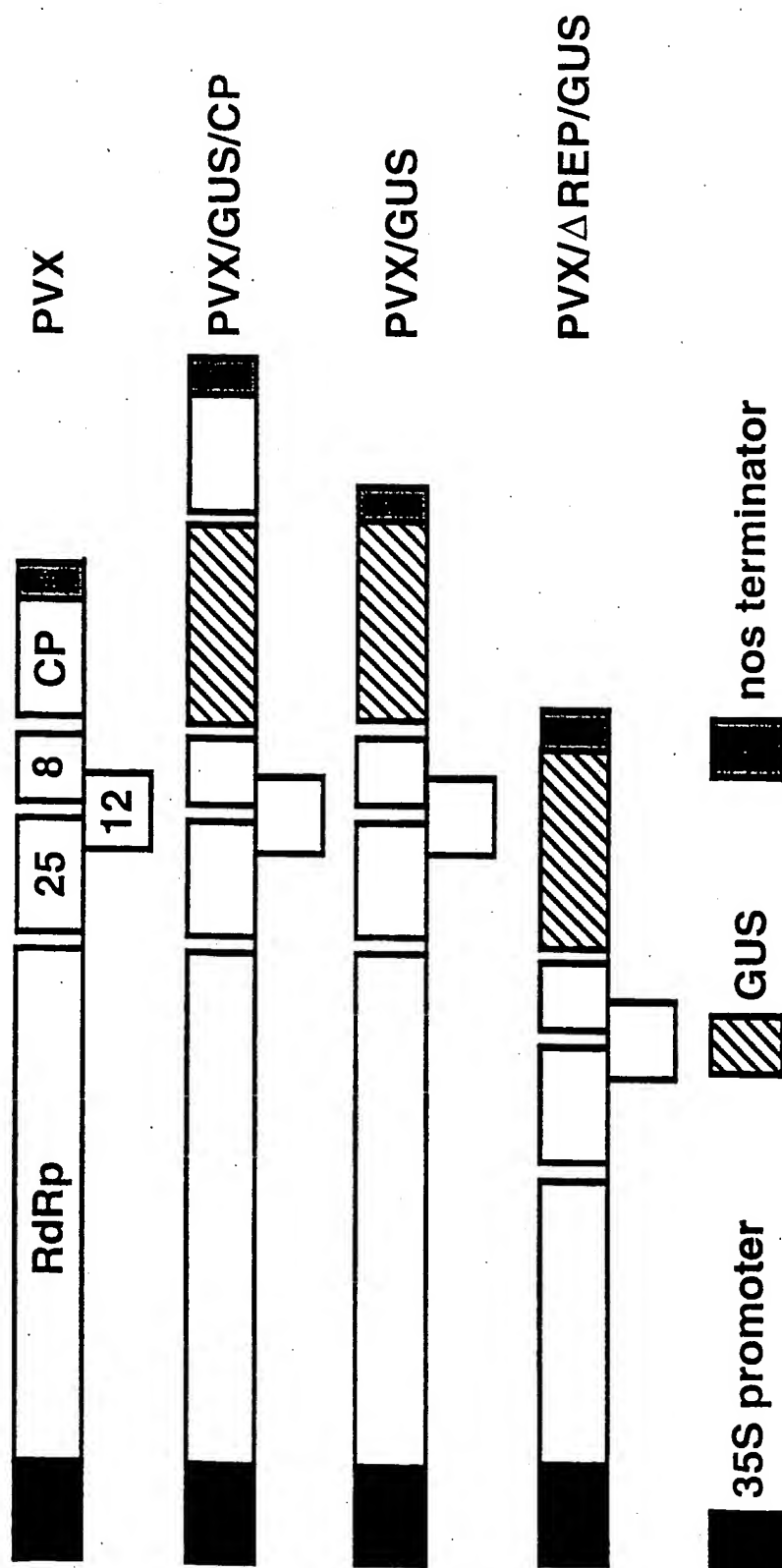


Figure 1

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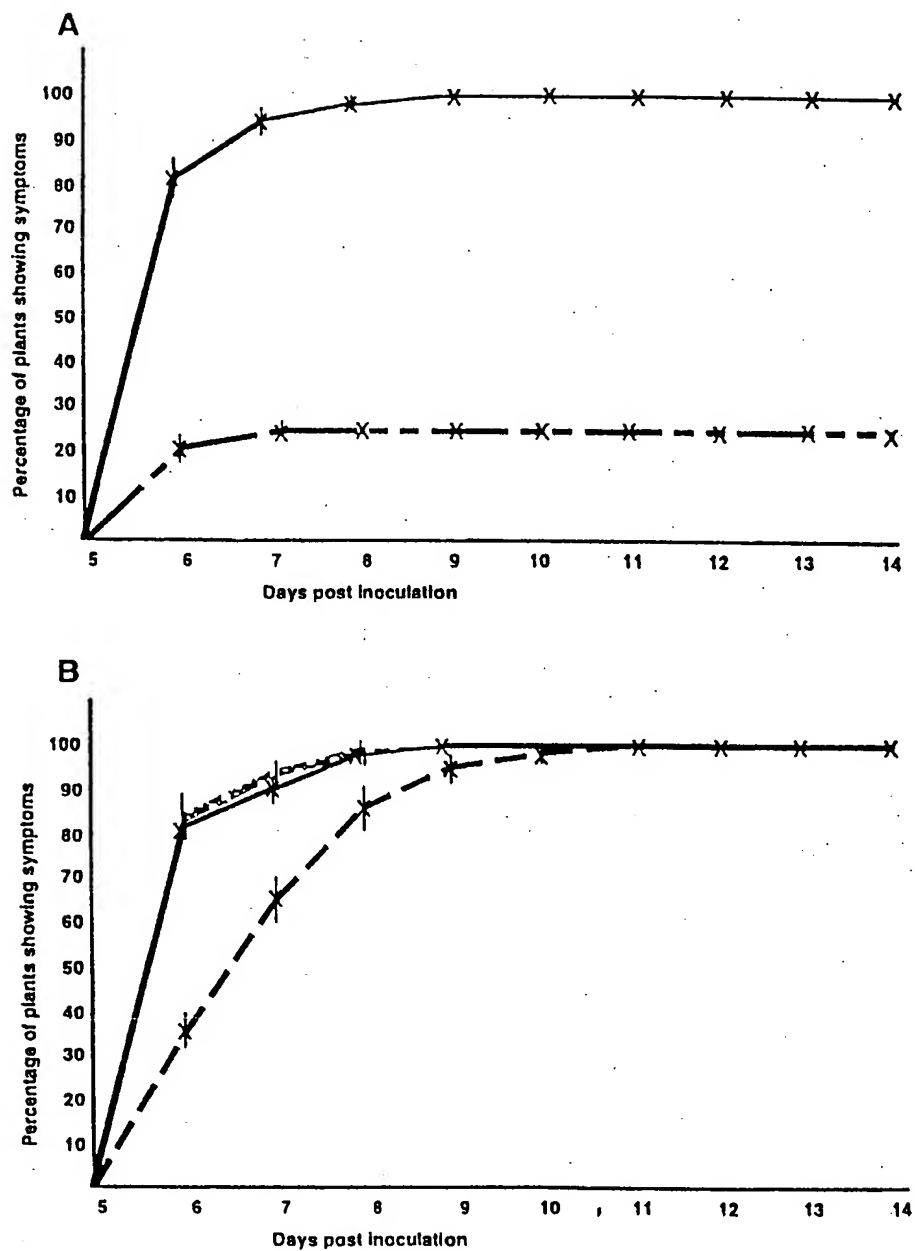


Figure 2

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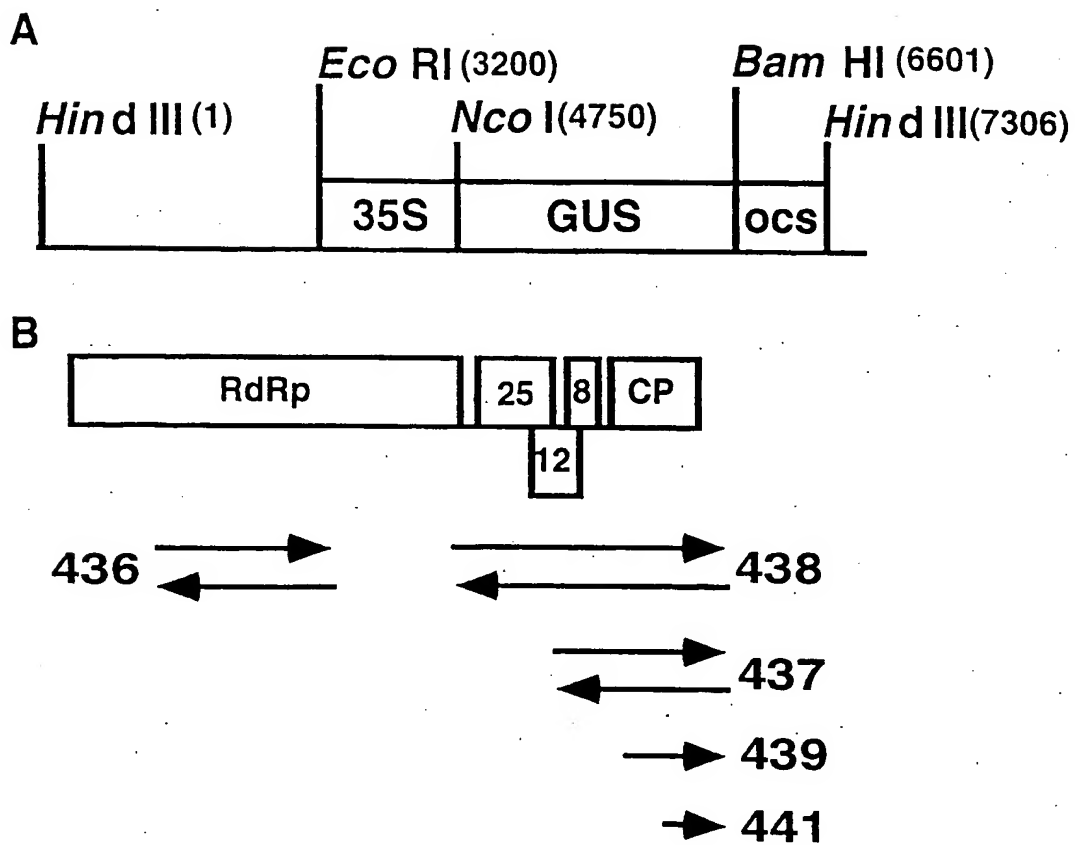


Figure 3

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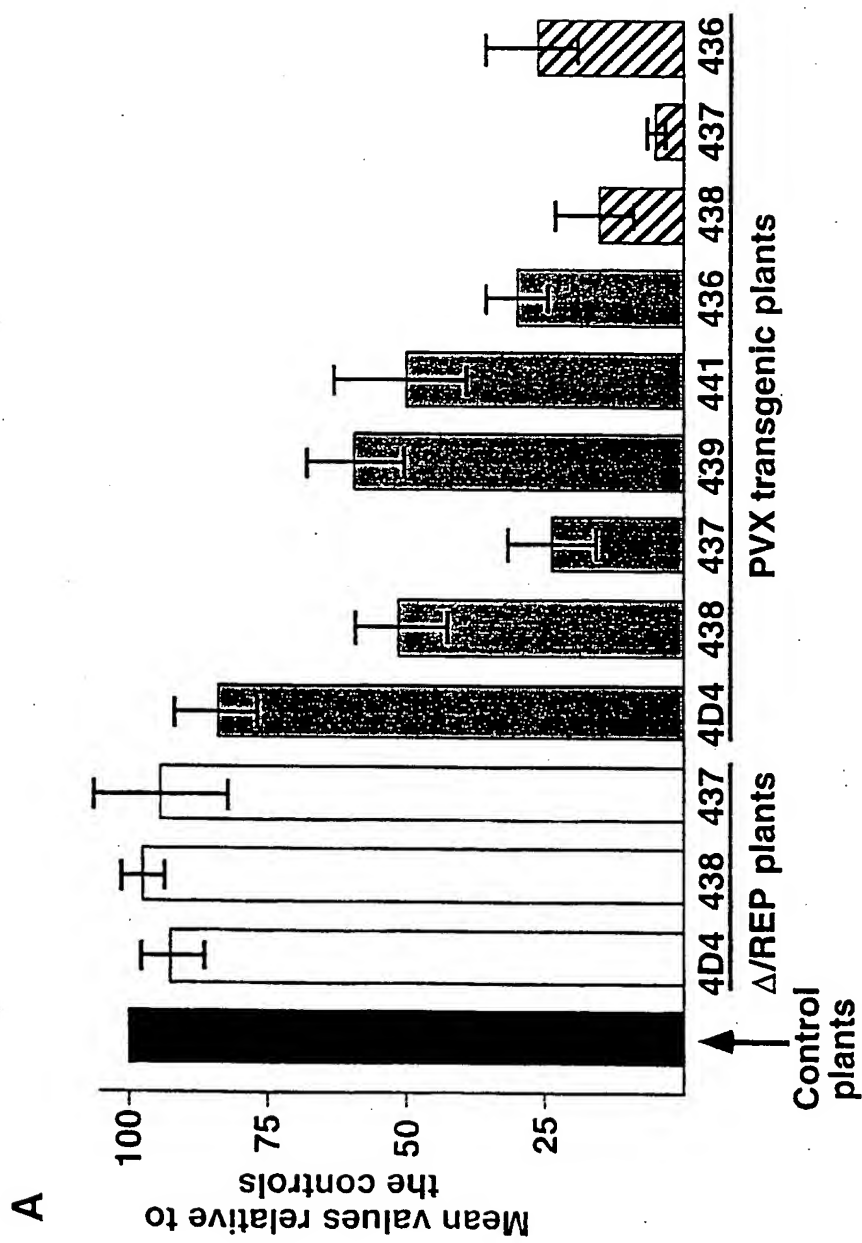


Figure 4

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Northern analysis of RNA extracted
from amplicon X GUS plants

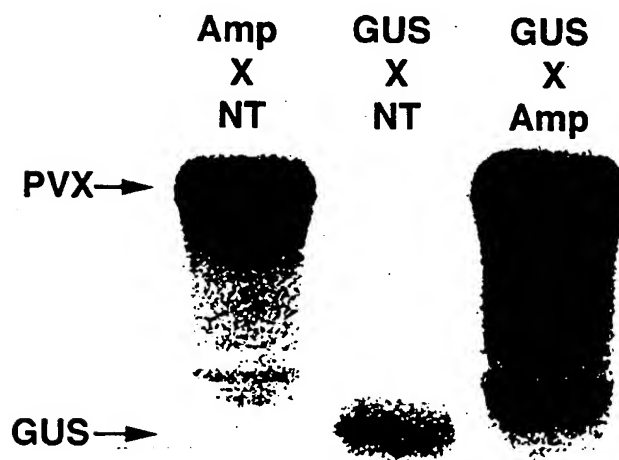


Figure 5

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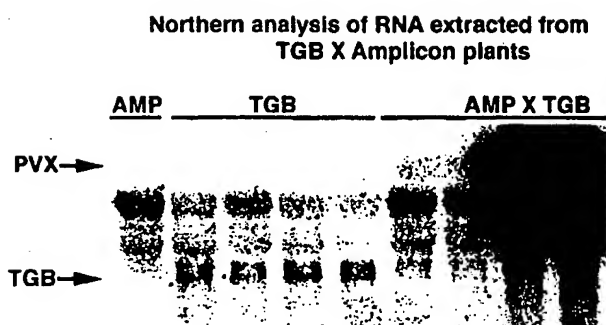


Figure 6

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Northern analysis of RNA
extracted from 35S/GUS
scions 5 weeks post grafting

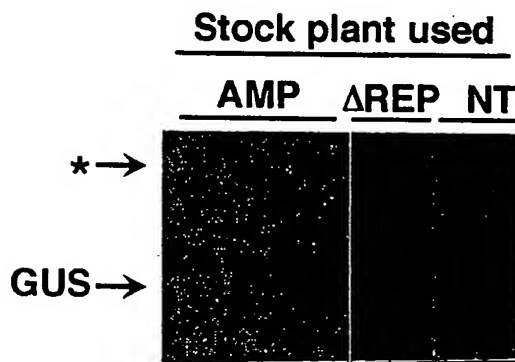


Figure 7

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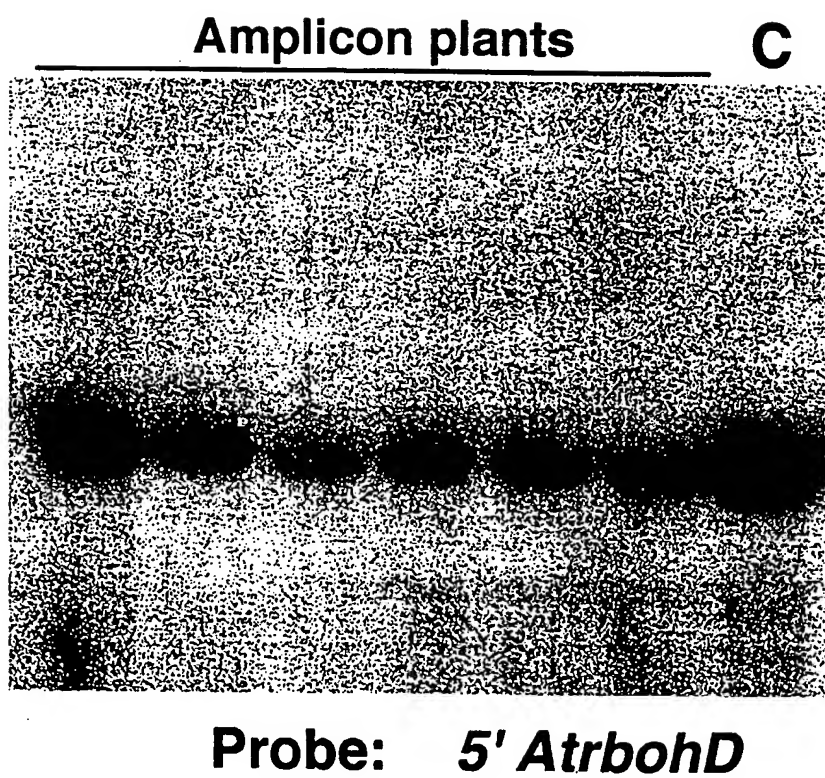


Figure 8

INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 98/00442

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/82 A01H3/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P	<p>ANGELL S.M. AND BAULCOMBE D.C.: "Consistent gene silencing in transgenic plants expressing a replicating potato virus X RNA" EMBO JOURNAL, vol. 16, no. 12, 1997, EYNHAM, OXFORD GB, pages 3675-3684, XP002067897 see the whole document</p> <p style="text-align: center;">--- -/--</p>	1-45



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

Special categories of cited documents:

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Date of the actual completion of the international search

12 June 1998

Date of mailing of the international search report

09/07/1998

Name and mailing address of the ISA

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Authorized officer

Panzica, G

INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 98/00442

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>WO 95 34668 A (BIOSOURCE TECH INC) 21 December 1995 cited in the application</p> <p>see abstract see page 1, line 5 - page 2, line 7 see page 3, line 9 - page 5, line 7 see page 7, line 31 - page 8, line 21 see page 9, line 15 - page 10, line 30 see claims</p> <p style="text-align: center;">---</p>	<p>1-5, 9-13, 18, 21-25, 27, 31-41</p>
X	<p>WO 95 25801 A (FOSTER GARY DAVID ; UNIVERSITY OF LEICESTER (GB)) 28 September 1995</p>	<p>1-11, 24, 25, 27-29</p>
Y	<p>see the whole document</p> <p style="text-align: center;">---</p>	<p>43-45</p>
Y	<p>WO 97 02352 A (CIBA GEIGY AG) 23 January 1997 see abstract see page 6, line 10 - page 11, line 9</p> <p style="text-align: center;">---</p>	<p>43-45</p>
X	<p>EP 0 425 004 A (CLOVIS MATTON N V ; AVEVE N V; SOLVAY & CIE SA) 2 May 1991 see abstract see page 6, line 9 - line 14 see page 7, line 15 - line 24</p> <p style="text-align: center;">---</p>	<p>1, 2, 4-6, 9, 10</p>
A	<p>see claims 1-3, 7, 11</p> <p style="text-align: center;">---</p>	<p>41-45</p>
A	<p>WO 91 13994 A (COMMW SCIENT IND RES ORG) 19 September 1991</p> <p style="text-align: center;">---</p>	<p>1, 19, 20, 41-45</p>
A	<p>BAULCOMBE D.C. AND ENGLISH J.J.: "Ectopic pairing of homologous DNA and post-transcriptional gene silencing in transgenic plants" CURRENT OPINION IN BIOTECHNOLOGY, vol. 7, no. 2, April 1996, LONDON, GB, pages 173-180, XP002067898 see the whole document</p> <p style="text-align: center;">---</p>	<p>1-45</p>
A	<p>LINDBO J.A. ET AL.: "Induction of a highly specific antiviral state in transgenic plants: implication of regulation of gene expression and virus resistance" PLANT CELL., vol. 5, December 1993, MD US, pages 1749-1759, XP002067899 cited in the application see the whole document</p> <p style="text-align: center;">---</p>	<p>41-45</p>
	-/--	

INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 98/00442

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>FINNEGAN J ET AL: "TRANSGENE INACTIVATION: PLANTS FIGHT BACK" BIO/TECHNOLOGY, vol. 12, September 1994, pages 883-888, XP002043134 cited in the application -----</p>	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/GB 98/00442

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9534668 A	21-12-1995	AU 2653495 A CA 2193094 A EP 0804600 A JP 10501968 T ZA 9504451 A	05-01-1996 21-12-1995 05-11-1997 24-02-1998 05-02-1996
WO 9525801 A	28-09-1995	AU 1954995 A	09-10-1995
WO 9702352 A	23-01-1997	AU 6358896 A	05-02-1997
EP 0425004 A	02-05-1991	NL 8902452 A NL 9001711 A AT 142700 T CA 2026703 A DE 69028479 D DE 69028479 T DK 425004 T ES 2094744 T IE 76133 B JP 3280883 A PT 95494 A,B	01-05-1991 01-05-1991 15-09-1996 04-04-1991 17-10-1996 03-04-1997 10-03-1997 01-02-1997 08-10-1997 11-12-1991 13-09-1991
WO 9113994 A	19-09-1991	NONE	

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